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13. ABSTRACT (Maximum 200 Words) The goal of this project is to elucidate the mechanisms of interactive effects of combined exposure to pyridostigmine bromide (PB), DEET and permethrin. The results presented in this final report, confirm our hypothesis that exposure to mixture of chemicals reduces the body's ability for their detoxification, resulting in increased bioavailability of the parent compounds, leading to pronounced toxicity. Loco-motor and sensorimotor deficits induced by single test compounds, were increased following binary or tertiary chemical exposures. Combined exposure to test compounds increased the permeability of the blood brain barrier (BBB) and caused neuronal death, that were exacerbated by stress, in the following brain regions: cingulate cortex, dentate gyrus, thalamus, and hypothalamus. Stress and combined exposure to test chemicals, also caused neuronal cell death in the following areas of the brain that showed no BBB disruption: the cerebral cortex, hippocampus, and the cerebellum. A major role for oxidative in the stress mechanisms of cell neuronal death reported in this report is supported by increased generation of 8-hydroxy-2-deoxyguanesis, 3-nitrolycyrosine, and cytochrome c by test compounds. Combined chemical exposure and stress caused testicular germ-cell apoptosis and liver damage in treated rats. Combined exposure to test compounds increased their bioavailability because of liver damage and competition for the detoxification enzymes in plasma and liver.				
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Introduction

A) The Nature of the Problem

The goal of this project is to evaluate the mechanisms of interactive effects of exposure to the insect repellent, DEET, the insecticide, permethrin, and anti-nerve agent, pyridostigmine bromide (PB) and the biological and pathological consequences of such interactions. The hypothesis to be tested is that combined exposure to a mixture of chemicals would have enhanced, and in some cases detrimental toxicological effects than exposure with single chemical as result of diminished ability of the body to detoxify these chemicals, and as a consequence, an increased effective concentration of the parent compound(s) in the circulation. The resultant toxic response is probably due to enhanced delivery and availability of individual chemicals to the target organ, and to specific molecules.

B) Approach

Our hypothesis is that interactions between combined chemical exposure would result in greater toxicological and pathological changes, and certain environmental modifying factors such as stress may exacerbate the toxic effects in combined exposure scenario. In order to investigate these toxic interactions we have utilized a various experimental modalities. The experimental approaches include neurobehavioral, neurochemical, pathological, and analytical methodologies. We have carried out dose-response studies ranging from 0.1-10 x the estimated human exposure of DEET, permethrin and PB. In our studies, we carried out neurobehavioral evaluations comprising a battery of well established locomotor and sensorimotor tests as well as neurochemical assessment, that included acetylcholinesterase, choline acetyl transferase, and nicotinic and muscarinic acetylcholine receptors following exposure to a single or multiple chemicals. Blood-brain barrier (BBB) permeability was evaluated by brain uptake of [³H]hexamethonium iodide uptake, and also by immunohistochemical staining for horseradish peroxidase. In the pathological studies, we have evaluated the extent of neurodegeneration within the motor cerebral cortex, the dentate gyrus, the CA1 and CA3 subfields of hippocampus, and the cerebellum following dermal application of DEET and permethrin, alone and in combination. Additionally, we studied the effect of one environmental modifier, stress on the neurotoxicity associated with concurrent exposure to PB, DEET and permethrin. The pathological changes were studied by immunohistochemical evaluation of microtubule-associated protein-2 (MAP-2), glial fibrillary acidic protein (GFAP), and microglial activation by lectin binding and BBB permeability using EBA immunostaining. Furthermore, we evaluated the possible mechanism(s) of neurotoxic effects of single or combined exposure by assessing the levels of oxidative stress. The levels of oxidative stress were evaluated by HPLC determination of 8-hydroxy-2'-deoxyguanosine, 3-nitrotyrosine and release of cytochrome c from brain mitochondria.

In order to perform comprehensive pharmacokinetics and bioavailability studies, we established the analytical methodology for simultaneous evaluation of DEET, permethrin,

and PB, and their metabolites by HPLC. Bioavailability of test compounds, DEET, permethrin and PB were evaluated under following conditions: a). Oral bioavailability of PB, alone or administered concurrently with dermal DEET and/or dermal permethrin. b). Dermal bioavailability of DEET, alone or administered concurrently with oral PB and/or dermal permethrin. c). Dermal bioavailability of permethrin, alone or administered concurrently with oral PB and/or dermal DEET. The data generated from all these studies have resulted in several peer-reviewed publications (**See the manuscripts in Appendix**).

Background

Combined chemical exposure to a variety of different chemicals can occur due to the widespread use of chemicals such as medications, food additives, and pesticides; many individuals may be exposed to these chemicals simultaneously. Interactions between the chemicals can lead to additive, synergistic or antagonistic effects. The mechanism of toxic interaction can be due to: a) toxicodynamic chemical interaction that can result in alterations of the response of the toxicity target, and b) toxicokinetic chemical interactions that result from alterations in the delivery of chemicals their site(s) of toxic action (1).

CNS cyto-architecture is maintained by a complex cellular milieu that involves neurons and a variety of cells of astrocytic and glial origin. In order for the CNS to function properly and to respond to external stimuli, it is absolutely required that a proper communication is maintained within these cells. A major determinant of neuronal morphology is the cytoskeleton. Different components of cytoskeleton within the neurons and astrocytes provide forces to maintain the appropriate cellular structure, e.g. neuronal dendrites and axons are maintained in stable conditions by the force provided by the elements of cytoskeleton (2). Such interactions are essential for proper synapse formation. The components of cytoskeleton are microfilament, intermediate filaments and microtubules. An important neuronal component, MAP-2 is enriched in dendrites and cell bodies (3), in which it stabilizes the polymerized tubulin. Abnormal regulation of expression of MAP-2 causes suppression of neurite outgrowth and reduction in number of neurites in cultured neurons (4). Similarly, aberrant intermediate filament proteins have been linked to diseases of neurodegeneration (5). A major component of astrocytic intermediate filament, GFAP is upregulated in response to reactive gliosis as a consequence of a variety of insults, such as exposure to neurotoxic chemicals, trauma, and neurodegenerative diseases that affect the CNS (6). The function of GFAP is not well understood, but it has been suggested to play important role in long-term maintenance of brain cyto-architecture (7), proper function of BBB (8), and modulation of neuronal functions (9).

Microglia are involved in brain function under both normal and pathological conditions (10, 11). In normal brain, resident ramified/resting microglia are activated to become rod-shaped or amoeboid shape in response to injury or toxic insult. Activated microglia proliferate, engulfing degenerating elements (12, 13), while secreting cytotoxic agents that induce neuronal death and demyelination (14, 15).

Oxidative stress resulting from environmental toxicants has been considered as a cause of chemical exposure related diseases. In the CNS, oxidative DNA damage has been observed following ischemia (16). Increased formation of 8-hydroxy-2'-deoxyguanosine has been reported in response to exposure with environmental toxicants (17, 18). Thus, it is apparent that a diverse mechanism could be activated in response to toxic insult that may result in pathological changes.

Test Compounds:

In our studies, we chose to study PB, DEET, and permethrin because our previous results have demonstrated that a combination of these chemicals causes greater neurobehavioral and neurotoxic changes than each chemical alone (19, 20, 21). In addition, thousands of U.S. Army personnel were presumably exposed to a combination of these chemicals during Persian Gulf War, and therefore, these studies may have human relevance.

Pyridostigmine Bromide (PB): PB is a quaternary dimethyl carbamate that has been used for the treatment of myasthenia gravis (22). It was given to veterans for prophylactic protection to shield acetylcholinesterase (AChE) from the nerve agent poisoning by reversibly inhibiting 30-40% of the AChE in the peripheral nervous system, thus protecting the enzyme from irreversible inhibition by nerve agents (23). The enzyme activity is restored following spontaneous decarbamylation resulting in near normal neuromuscular and autonomic functions (23). Toxic symptoms associated with PB overdose are primarily associated with over-stimulation of nicotinic and muscarinic receptors in the peripheral nervous system resulting in exaggerated cholinergic effects such as muscle fasciculations, cramps, weakness, muscle twitching, tremors, respiratory difficulty, gastro-intestinal tract disturbances and paralysis (24). The major metabolic product of PB is 3-hydroxy-*N*-methylpyridinium resulting from the carbamate hydrolysis that abolishes its cholinergic action (25, 26). Central nervous system effects of PB are not observed unless blood-brain barrier (BBB) permeability is compromised, because PB does not cross the BBB owing to the positive charge on the quaternary pyridinyl nitrogen (27).

***N,N*-diethyl-*m*-toluamide (DEET):** DEET is commonly used as an insect repellent against mosquitoes, flies, ticks and other insects in the form of lotion, stick or spray (28, 29). Extensive and repeated topical application of DEET resulted in human poisoning including death (30, 31, 32). The symptoms associated with DEET poisoning are characterized by tremors, restlessness, difficulty in speech, seizures, impairment of cognitive function and coma (29). Extremely high levels of DEET exposure has been reported to cause spongiform myelinopathy (33). Because DEET efficiently crosses the dermal barrier (34, 35) and localizes to dermal fat deposits (36, 37), it is possible that DEET may enhance the availability of drugs and toxicants in other organs and cause regulatory changes such as changes in blood brain permeability. However, it is not known with certainty that DEET could enhance the neurotoxicity associated with permethrin or PB because of its lipophilic nature.

Permethrin: Permethrin is a type I synthetic pyrethroid insecticide that exists in four different stereoisomers (38). Its insecticidal activity persists for several weeks following a single application. Permethrin intoxication results as a consequence of modification of sodium channel such that it remains open for a longer time, leading to repetitive discharges after single stimulus (39). This repetitive nerve action is associated with tremors, hyperactivity, ataxia, convulsions, and in some cases to paralysis. It has been reported that high dose of permethrin given to rats induced peripheral nerve damage through its effects on sodium channels (40).

BODY

Key Research Accomplishments

The goal of this project is to characterize and elucidate interactive effects of combined exposure to the anti-nerve gas agent prophylaxis, pyridostigmine bromide (PB); the insect repellent, DEET; and the insecticide, permethrin. This goal is to be accomplished through carrying out the following studies:

- A. Neurotoxic effects of PB, DEET, and permethrin, alone and in combination
- B. Effect of the test compounds in the permeability of the blood-brain barrier
- C. Effect of the test compounds on testicular germ-cell apoptosis
- D. Bioavailability, Pharmacokinetics and Metabolism of test compounds

We have carried out the following studies to accomplish the project's objective.

A. Neurotoxic effects of pyridostigmine bromide (PB), DEET, and permethrin, alone and in combination.

1. Locomotor and sensorimotor performance

a. Effects of Daily Dermal Application of DEET and Permethrin, Alone and in Combination, on Sensorimotor Performance

Groups of five rats were treated with a dermal daily dose of 4, 40, or 400 mg/kg DEET in ethanol or 0.013, 0.13, or 1.3 mg/kg permethrin in ethanol for 60 d. A group of five rats received a daily dermal dose of ethanol and served as a control. Sensorimotor ability was assessed by a battery of behavioral tests that included beam-walk score, beam-walk time, incline plane performance, and forepaw grip on days 30, 45, and 60 days after exposure. All treatments caused a significant decline in sensorimotor performance in a dose- and time-dependent manner. There was no effect of any of the test chemical alone or in combination, on simple sensorimotor reflexes. These studies show that DEET and permethrin, alone or in combination caused a dose-dependent deficit in sensorimotor performance even at one-tenth the real-life exposure dose. Elevating dose and longer duration of exposure caused increasingly greater deficits (19).

b. Locomotor and Sensorimotor Performance Deficit in Rats following Exposure to PB, DEET, and Permethrin, Alone and in Combination

i. 1x Dose: Male Sprague-Dawley rats (200-250 gm) were treated daily with DEET (40 mg/kg, dermal) or permethrin (0.13 mg/kg, dermal), alone and in combination with PB (1.3 mg/kg, oral, last 15 days only), for 45 days. Sensorimotor ability was assessed by a battery of behavioral tests that included beam-walk score, beam-walk time, incline plane performance, and forepaw grip on days 30 and 45 following the treatment. On day 45 the animals were sacrificed. Animals treated with PB, alone or in combination with DEET

and permethrin, showed a significant deficit in beam-walk score as well as beam-walk time as compared with controls. Treatment with either DEET or permethrin, alone or in combination with each other, did not have a significant effect on beam-walk score. All chemicals, alone or in combination, resulted in a significant impairment in incline plane on days 30 and 45 following treatment.

ii) *Combination of 0.1x, 1x and 10x dose:* Male Sprague-Dawley rats (200-250 gm.) were treated with either PB (0.13, 1.3 or 13 mg/kg, oral by gavage) or a combination of PB (0.13, 1.3 or 13 mg/kg, oral by gavage) and DEET (4, 40, and 400 mg/kg, dermal), PB (0.13, 1.3 or 13 mg/kg, oral by gavage) and permethrin (0.013, 0.13 and 1.3mg/kg, dermal) or PB in combination with DEET and permethrin at these doses. Rats were treated with DEET and permethrin daily for 60 days whereas PB was given on only the last 15 days. Sensorimotor ability was assessed by a battery of behavioral tests that included beam-walk score, beam-walk time, incline plane performance and fore-paw grip on day 60 following the beginning of the treatment with DEET and permethrin. Treatment with PB alone or in combination with DEET, or in combination with DEET+ permethrin resulted in deficit in beam-walk score as well as beam-walk time as compared with controls. Similarly, PB alone, in combination with DEET, or in combination with DEET + permethrin resulted in an impairment in incline plane performance. All treatments impaired forepaw grip strength.

2. Plasma Butyrylcholinesterase (BChE), Acetylcholinesterase (AChE) and Choline Acetyl Transferase (CHAT)

a. Effect of PB, DEET, and Permethrin, Alone and in Combination on Rat Plasma, BChE, Brain AChE, and Brain ChAT Activities

Male Sprague-Dawley rats (200-250 gm) were treated with DEET (40 mg/kg, dermal) or permethrin (0.13 mg/kg, dermal), alone and in combination with PB (1.3 mg/kg, oral, last 15 days only), for 45 days (20). On Day 45 the animals were sacrificed, and the activities of plasma and brain cholinesterases, and brain ChAT were determined. Treatment with PB, DEET, or permethrin alone did not have any inhibitory effect on plasma or brain cholinesterase activities, except that PB alone caused moderate inhibition in midbrain AChE activity. Treatment with permethrin alone caused significant increase in cortical and cerebellar AChE activity. Although AChE is not a direct target of permethrin, its effects on AChE activity may be the result of an indirect regulation resulting in the increased gene regulation of AChE; this needs further studies. A combination of DEET and permethrin or PB and DEET led to significant decrease in AChE activity in brainstem and midbrain and brainstem, respectively. A significant decrease in brainstem AChE activity was observed following combined exposure to PB and permethrin. Coexposure with PB, DEET, and permethrin resulted in significant inhibition in AChE in brainstem and midbrain. No effect was observed on ChAT activity in brainstem or cortex, except combined exposure to PB, DEET, and permethrin caused a slight but significant increase in cortical choline acetyltransferase activity.

b. Effect of Combined Exposure to Low Daily Doses of PB, DEET, and Permethrin, With and Without Stress

Two groups of 5 male Sprague-Dawley rats were administered in combination PB (1.3 mg/kg/d, oral), DEET (40 mg/kg/d, dermal), and permethrin (0.13 mg/kg/d, dermal) for 28 days. Rats in one of the above two groups were subjected to stress every day for the duration of the experiment by placing them in a Plexiglas restraint tube for 5 minutes. Two additional groups of animals (one subjected to stress and vehicle treatment, and another treated with vehicle alone) served as controls (41). Thus, out of a total of four groups of rats, two groups were subjected to stress; one in combination with three chemicals and the other vehicle treated. AChE in cerebral cortex, brainstem, midbrain, and cerebellum and BChE in plasma activities were assayed. Combined exposure to chemicals and stress caused a significant decrease in AChE activity in midbrain, brainstem, and cerebellum compared to control animals (~65%, 71%, and 64% of controls). Plasma BChE activity was also significantly inhibited (66% of control) by combined exposure to chemicals and stress. In addition, animals treated with chemicals and stress exhibited a significant decrease compared to animals treated with stress alone in midbrain, cerebellum, and plasma. Inhibition of brain AChE activity following treatment with a combination of stress and three chemicals is suggestive of PB penetrating in to the CNS, and thus mediating this inhibition. These results, however, are inconsistent from studies by Lallement et al (1998)(42), Tian et al (2002)(43), Grauer et al (2000)(44), Sinton et al (2000)(45), Kant et al (2001)(46), as these studies did not observe any inhibition of brain AChE activity by PB under a variety of stress conditions. The discrepancies between our findings and those of these aforementioned groups could arise from a variety of reasons, such as dose, stress, duration and animal species and age of the animal. In addition, there could be peripheral mechanisms that can affect the BBB permeability.

3. Ligand Binding for m2-Muscarinic (m2 mAChR) and Nicotinic (nAChR) Acetylcholine Receptors

a. Effect of PB, DEET, and Permethrin, Alone or in Combination

Male Sprague-Dawley rats (200-250 gm) were treated with DEET (40 mg/kg, dermal) or permethrin (0.13 mg/kg, dermal), alone and in combination with PB (1.3 mg/kg, oral, last 15 days only), for 45 days (19). On day 45 the animals were sacrificed. [³H]AF-DX 384 at 2.2 nM concentration was used as the ligand for m2 mAChR and [³H]cytisine at 1 nM concentration was used as the ligand for nAChR containing $\alpha 2\beta 4$ subunits. Treatment with PB, DEET, and permethrin alone caused a significant increase in ligand binding for m2- mAChR in the cortex. Co-exposure to PB, DEET, and permethrin did not have any effect over that of PB-induced increase in ligand binding. There was no significant change in ligand binding for nAChR associated with treatment with the chemical alone; a combination of PB and DEET or co-exposure with PB, DEET, and permethrin caused a significant increase in nAChR ligand binding in the cortex.

b. Effect of Combined Exposure to Low Daily Doses of PB, DEET, and Permethrin, With and Without Stress

Two groups of 5 male Sprague-Dawley rats were administered PB (1.3 mg/kg/d, oral), DEET (40 mg/kg/d, dermal), and permethrin (0.13 mg/kg/d, dermal) for 28 days. Animals in one of the above two groups were subjected to stress every day for the duration of the experiment by placing them in a Plexiglas restraint tube for 5 minutes. Two additional groups of animals (one subjected to stress and vehicle treatment, and another treated with vehicle alone) served as controls (41). Ligand binding studies were carried out for m₂-muscarinic receptor in cerebral cortex, midbrain, brainstem, and cerebellum using m₂-selective ligand, [³H] AFDX-384. Animals treated with stress alone exhibited a significant decrease compared to controls in cortex and brainstem. Animals treated with chemicals alone exhibited a significant decrease compared to animals treated with stress alone in midbrain. Animals treated with chemicals and stress exhibited a significant decrease compared to controls in cortex and cerebellum. Animals treated with chemicals and stress also exhibited a significant decrease compared to animals treated with stress alone in midbrain, and cerebellum.

4. Neuropathological Alterations

a. *Dermal Application of DEET and Permethrin to Adult Rats, Alone or in Combination*

Histopathological alterations were evaluated in the brain of adult male rats following a daily dermal dose of DEET (40 mg/kg in 70% ethanol) or permethrin (0.13 mg/kg in 70% ethanol) or a combination of the two for 60 days (47). Control rats received a daily dermal dose of 70% ethanol for 60 days. Animals were perfused and brains were processed for morphological and histopathological analyses following the above regimen. Quantification of the density of healthy (or surviving) neurons in the motor cerebral cortex, the dentate gyrus, the CA1 and CA3 subfields of the hippocampus, and the cerebellum revealed significant reductions in all three treated groups compared with the control group. Further, animals receiving either DEET or permethrin exhibited a significant number of degenerating (eosinophilic) neurons in the above brain regions. However, degenerating neurons were infrequent in animals receiving both DEET and permethrin, suggesting that neuronal cell death occurs earlier in animals receiving combined DEET and permethrin than in animals receiving either DEET or permethrin alone. The extent of neuron loss in different brain regions was similar among the three treatment groups except the dentate gyrus, where neurodegeneration was significantly greater with exposure to DEET alone. The neuron loss in the motor cerebral cortex and the CA1 subfield of all treated groups was also corroborated by a significant decrease in microtubule associated protein 2-immunoreactive elements (15-52% reduction), with maximal reductions occurring in rats receiving DEET alone; further, the surviving neurons in animals receiving both DEET and permethrin exhibited wavy and beaded dendrites. Analysis of glial fibrillary acidic protein (GFAP) immunoreactivity revealed significant hypertrophy of astrocytes in the hippocampus and the cerebellum of all treated groups (24-106% increase). Thus, subchronic dermal application of DEET and permethrin to adult rats, alone or in combination, leads to a diffuse neuronal cell death in the cerebral cortex, the hippocampal formation, and the cerebellum. Collectively, the

above alterations can lead to many physiological, pharmacological, and behavioral abnormalities, particularly motor deficits and learning and memory dysfunction.

b. Effect of Combined Exposures to Low Daily Doses of PB, DEET, and Permethrin, With and Without Stress

Two groups of 5 male Sprague-Dawley rats were administered PB (1.3 mg/kg/d, oral), DEET (40 mg/kg/d, dermal), and permethrin (0.13 mg/kg/d, dermal) for 28 days. Animals in one of the above two groups were subjected to stress every day for the duration of the experiment by placing them in a Plexiglas restraint tube for 5 minutes. Two additional groups of animals (one subjected to stress and vehicle treatment, and another treated with vehicle alone) served as controls (41). Animals treated with chemicals and stress exhibited neuronal cell death and significantly enhanced astrocytic GFAP expression (suggestive of glial hypertrophy) in many regions of the brain. The regions where these neuropathological alterations were conspicuous include the motor and somatosensory regions of the cerebral cortex, and the dentate gyrus and CA1 and CA3 subfields of the hippocampus. In cerebral cortex, the neuronal cell death was more pronounced in pyramidal neurons belonging to layers III and V of the motor cortex, pyramidal and granule neurons belonging to layers III and V of the somatosensory cortex, granule cells of the dentate gyrus, pyramidal neurons of hippocampal CA1 and CA3 subfields. In addition, these regions also demonstrated abnormalities in the expression of the cytoskeletal proteins such as MAP-2. The dendrites of surviving neurons clearly had reduced MAP-2 protein. The abnormalities in MAP-2 expression in some of the surviving neurons (particularly apical dendrites of cortical pyramidal neurons in layer III) were characterized by a beaded, disrupted, or wavy appearance. The GFAP expression in this group was exemplified by GFAP expression in soma of astrocytes and hypertrophy of astrocytic processes emanating from the soma. Thus, there is clearly a more pronounced neural injury following exposure to both chemicals and stress in combination than exposure to either chemicals or stress alone.

5. Effect on the Liver

a. Histopathological Changes in the Liver

Effect of Combined Daily Exposure to PB, DEET, and Permethrin, alone and with Stress.

Two groups of 5 male Sprague-Dawley rats were administered a combination of PB (1.3 mg/kg/d, oral), DEET (40 mg/kg/d, dermal), and permethrin (0.13 mg/kg/d, dermal) for 28 days. Rats in one of the above two groups were subjected to stress every day for the duration of the experiment by placing them in a Plexiglas restraint tube for 5 minutes. Two additional groups of animals (one subjected to stress and vehicle treatment, and another treated with vehicle alone) served as controls (41). Examination of sections through the liver stained with H&E showed mild alterations in hepatic cytoarchitecture, characterized by portal and peri-portal fibrosis with mononuclear inflammatory cells. In addition, sinusoidal dilatation, markedly diffuse steatosis, predominant microvacuoles were observed in animals treated with both chemicals and stress, in comparison to control

animals. Liver damage by combined test compounds and stress may explain the inhibition of plasma BChE since this enzyme is synthesized in the liver.

b. Effect on Hepatic Cytochrome P4503A

DEET alone and in combination, with permethrin increased urinary excretion of 6 β -hydroxycortisol in rats, a marker of hepatic CYP3A induction. In this study, the ratio of 6 β -hydroxycortisol (6 β -OHF) to free cortisol (F) was determined in urine following a single dermal dose of 400 mg/kg of DEET (*N, N*-diethyl *m*-toluamide), and 1.3 mg/kg of permethrin, alone and in combination, in rats (48). Urine samples were collected at 2, 4, 8, 16, 26, 48, and 72 h after application. Recoveries of 6 β -OHF and cortisol (F) from control urine samples were between 75 and 85% with limits of detection at 30 and 10 ng/ml for cortisol and 6 β -OHF, respectively. A single dermal dose of DEET alone and in combination with permethrin significantly increased urinary excretion of 6 β -hydroxycortisol. These results indicate that DEET, alone and in combination with permethrin, increased urinary excretion of 6 β -OHF in rats following a single dermal dose application. Furthermore, an increase in the urinary excretion of 6B-OHF suggests that acute treatment with DEET induced expression of CYP3A, a cytochrome P-450 involved in the metabolism of diverse array of xenobiotics.

c. Binding of PB, DEET and permethrin, alone and in combination to human serum albumin

In these studies, binding of PB, DEET and permethrin to human serum albumin was evaluated following incubation with 500ng-10 μ g/ml of PB, DEET and permethrin at 37°C for 60 minutes (49). The results showed that 81.2 \pm 4.2%, and 84.6 \pm 2.5% of the initial concentration of PB was bound to HAS when incubated alone or in combination with DEET or permethrin, respectively. DEET and permethrin did not show any significant interaction with HAS. There was no significant change in the binding to HAS when the incubation was carried out in the presence of three compounds.

6. Effect on Oxidative Stress Markers

a. Induction of Urinary Excretion of 3-Nitrotyrosine, a Marker of Oxidative Stress, following Administration of PB, DEET, and Permethrin, Alone and in Combination in Rats

Levels of 3-nitrotyrosine in rat urine were determined following administration of a single oral dose of 13 mg/kg PB, a single dermal dose of 400 mg/kg DEET and a single dermal dose of 1.3 mg/kg permethrin, alone and in combination (50). Urine samples were collected from five treated and five control rats at 4, 8, 16, 24, 48, and 72 h following dosing. Solid-phase extraction coupled with high-performance liquid chromatography with ultraviolet detection at 274 nm was used for the determination of tyrosine and 3-nitrotyrosine. A single oral dose of PB and a single dermal dose of DEET or their combination significantly ($P < 0.05$) increased levels of 3-nitrotyrosine starting 24 hrs after dosing compared with control urine samples. The maximum increase of 3-nitrotyrosine was detected 48 h after combined administration of PB and DEET. The ratio of 3-nitrotyrosine to tyrosine in urine excreted 48 h after dosing was $0.19 \pm$

0.04, 0.20 ± 0.05 , 0.28 ± 0.03 , 0.32 ± 0.04 , 0.19 ± 0.05 , 0.42 ± 0.04 , 0.27 ± 0.03 , 0.36 ± 0.04 , and 0.48 ± 0.04 following administration of water, ethanol, PB, DEET, permethrin, PB + DEET, PB + permethrin, DEET + permethrin, and PB + DEET + permethrin, respectively. The results indicate that an oral dose of PB and a dermal administration of DEET, alone and in combination, could generate free radical species, and thus increase levels of 3-nitrotyrosine in rat urine. Induction of 3-nitrotyrosine, a marker of oxidative stress, following exposure to these compounds could be significant in understanding the proposed enhanced toxicity following combined exposure to these compounds.

b. Combined exposure to DEET and Permethrin-Induced Release of Rat Brain Mitochondrial Cytochrome c

The release of cytochrome c from the mitochondrial intermembrane space can induce apoptosis. The levels of mitochondrial cytochrome c in rat brain following a single dermal dose of 400 mg/kg of DEET, and of 1.3 mg/kg of permethrin, alone or in combination were determined (51). Rats were sacrificed at a time interval of 0.5, 1, 2, 4, 8, 16, 24, 48, or 72 h after dosing. Brain mitochondria were isolated and the levels of cytochrome c were measured using reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. Average percentage recovery cytochrome c spiked with control rat brain mitochondria was $83.2 \pm 8.9\%$. Limits of detection and quantitation were 1 and 5 ng, respectively. The results showed that a single dermal dose of a combination of DEET and permethrin significantly increased the release of brain mitochondrial cytochrome c starting 24 h after treatment. DEET and permethrin alone did not affect the release of cytochrome c. The results indicate that combined exposure to DEET and permethrin might induce the apoptotic processes in rat brain as seen by the release of cytochrome c.

B. Effect of Test Compounds on the Permeability of the Blood-Brain Barrier

1. Effects of Daily Dermal Application of DEET and Permethrin, Alone and in Combination, on Blood-Brain Barrier

Brain Regions uptake of [3 H]hexamethonium Iodide.

This study was designed to investigate the effects of repeated daily dermal application of DEET and permethrin, alone or in combination on the integrity of the BBB in male Sprague-Dawley rats (19). The route of exposure and the dose levels of test compounds were chosen to closely reflect those present during the Gulf War. Both test compounds were applied dermally using 0.1, 1, and 10 times the estimated real life doses of 40 mg/kg/d for DEET and 0.13 mg/kg/d for permethrin. The data suggest that physiologically relevant exposure to DEET for 60 d caused a decrease in BBB permeability in the brainstem at 1 x and 10 x doses. Permethrin exposure alone had no observable effect on the BBB permeability. However, a combination of the two chemicals caused a decrease in the BBB permeability in the cortex and in a dose-dependent fashion as compared to DEET or permethrin alone, suggesting that the two chemicals together may have additive effects, but only in selected brain region(s) such as

cortex. The decrease in BBB permeability with combined exposure does not necessarily reflect an antagonistic effect. It could also mean that both chemicals in combination cause changes in cerebrovascular endothelium, leading to a decrease in [^3H]hexamethonium iodide uptake. The decrease in BBB permeability could be mediated by one of several mechanisms; for example, it is known that membrane fluidity changes induced by ethanol cause a decrease in the passage of [^3H]vincristine into the CNS. A decrease in blood flow to the CNS could also cause a reduction in the availability of [^3H]hexamethonium iodide to the CNS. These results suggest that DEET exposure alone or in combination with permethrin by dermal application can lead to BBB permeability changes in certain brain region(s) that can have important physiological/pharmacological consequences.

2. Combined Exposure to Low Daily Doses of PB, DEET, and Permethrin, With or Without Stress

a. Brain Regions Uptake of [^3H]hexamethonium Iodide

Two groups of 5 male Sprague-Dawley rats were administered PB (1.3 mg/kg/d, oral), DEET (40 mg/kg/d, dermal), and permethrin (0.13 mg/kg/d, dermal) for 28 days. Animals in one of the above two groups were subjected to stress every day for the duration of the experiment by placing them in a Plexiglas restraint tube for 5 minutes. Two additional groups of animals (one subjected to stress and vehicle treatment, and another treated with vehicle alone) served as controls (41). The effect of combined daily exposure to PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal) and permethrin (0.13 mg/kg, dermal) for 28 days with and without stress on BBB permeability was evaluated. Exposure to either chemicals or stress alone had no significant effect on BBB permeability. In contrast, combined exposure to chemicals and stress led to a significant increase in BBB permeability in cerebral cortex, cerebellum and the midbrain. The increase in BBB permeability in cerebellum was ~366% of control animals, suggesting that cerebellum is the most susceptible brain area for BBB disruption following exposure to the combination of chemicals and stress, as assessed by [^3H]hexamethonium iodide uptake.

b. Histological Assessment of Horseradish Peroxides Penetration

BBB permeability was also assessed in other groups of 5 animals as described above by the penetration of horseradish peroxidase (HRP) into the parenchyma of the CNS as vesicles positive for HRP using DAB staining (41). Exposure to chemicals or stress alone resulted in minimal perivascular penetration of HRP. Whereas, co-exposure with chemicals and stress resulted in abundant HRP positive vesicles in cerebral cortex, cerebral white matter such as corpus callosum, deep gray nuclei and brain stem compared to animals treated with chemicals alone.

A dense vesicular staining of HRP in the cytoplasm, abluminal surface, basement membrane, extra-cellular spaces of adjacent neuropil, and endothelial tight junctions in animals treated with both chemicals and stress were evident. Whereas, in animals

subjected to stress alone, only a moderate HRP-positive staining was observed. In contrast, in control animals there was a complete absence of HRP-positive staining.

Since increased BBB permeability results in microglial activation and hypertrophy, the hypertrophy of microglial cells was investigated by immunohistochemical detection of lectin. A very extensive lectin binding around capillaries and prominent patches of diffuse lectin staining were evident in the brain parenchyma of animals exposed to both chemicals and stress. Furthermore, processes of microglial cells in animals exposed to combination of stress and chemicals exhibited increased complexity, suggesting that microglial cells undergo hypertrophy following exposure to both chemicals and stress.

C. Effect of the test compounds on testicular germ-cell apoptosis

Testicular germ-cell apoptosis was studied following subchronic exposure of male, Sprague-Dawley rats to combined doses of pyridostigmine bromide (PB, 1.3 mg/kg/d in water, oral); the insect repellent *N,N*, -diethyl *m*-toluamide (DEET, 40 mg/kg/d in ethanol, dermal), and the insecticide permethrin (0.13 mg/kg in ethanol, dermal), with and without stress, daily for 28 days. Exposure to stress alone produced no remarkable histopathological alterations in the testes. Administration of combination of these chemicals induced apoptosis in rat testicular germ cells, Sertoli cells and Leydig cells, as well as the endothelial lining of the blood vessels. Testicular damage was significantly augmented when the animals were further subjected to stress. Histopathological examination of testicular tissue sections showed that apoptosis was confined to the basal germ cells, and spermatocytes, indicating suppression of spermatogenesis. Increased apoptosis of testicular cells coincided, in timing and localization, with increased expression of the apoptosis-promoting proteins Bax and p53. Furthermore, significant increase of 3-nitrotyrosine immunostaining in the testes revealed oxidative and/or nitrosation induction of cell death (52).

D. Bioavailability, Metabolism and Pharmacokinetic Studies

1. Development of Analytical Methods for Test Compounds and Their Metabolites

Simultaneous Determination of PB, DEET, Permethrin, and Their Metabolites in Rat Plasma and Urine by High-performance Liquid Chromatography

A rapid and simple method was developed for the separation and quantification of the anti nerve agent drug PB, its metabolite *N*-methyl-3-hydroxypyridinium bromide, the insect repellent DEET, its metabolites *m*-toluamide and *m*-toluic acid, the insecticide permethrin, and two of its metabolites *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid in rat plasma and urine (53). The method is based on using C₁₈ Sep-Pak[®] cartridges for solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) with reversed-phased C₁₈ column, and gradient UV detection ranging between 208 and 230 nm. The compounds were separated using gradient of 1 to 99% acetonitrile in water (pH 3.20) at a flow-rate ranging between 0.5 and 1.7 ml/min in a period of 17 min. The retention times ranged from 5.7 to 14.5 min. The limits of detection were ranged

between 20 and 100 ng/ml, while limits of quantitation were 150-200 ng/ml. Average percentage recovery of five spiked plasma samples were 51.4 ± 10.6 , 7.1 ± 11.0 , 83.2 ± 6.7 , 60.4 ± 11.8 , 63.6 ± 10.1 , 69.3 ± 8.5 , 68.3 ± 12.0 , 82.6 ± 8.1 , and from urine 55.9 ± 9.8 , 60.3 ± 7.4 , 77.9 ± 9.1 , 61.7 ± 13.5 , 68.6 ± 8.9 , 62.0 ± 9.5 , 72.9 ± 9.1 , and 72.1 ± 8.0 , for PB, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid, respectively. The relationship between peak areas and concentration was linear over the range between 100 and 5000 ng/ml. This method was applied to analyze the above chemicals and metabolites following their administration in rats.

2. Bioavailability of Test Compounds

a. Oral Bioavailability of PB, Alone or When Administered concurrently with Dermal DEET and/or Dermal Permethrin

To determine the oral bioavailability of PB two groups of 40 male Sprague-Dawley rats were given a single intravenous (i.v) dose of 1.3 mg/kg PB in saline or an oral dose (po) of 13 mg/kg PB in water. To determine the oral bioavailability of PB with concurrent exposure to other chemicals, three groups of 40 rats were treated with an oral dose of 13 mg/kg PB, then one of these groups was treated with a dermal dose of 400 mg/kg DEET, a dermal dose of 1.3 mg/kg permethrin, or a combination of both. Dermal application was carried out in ethanol, on an unprotected 1 cm² area of the pre-clipped skin on the back of the neck. At each of the following time intervals, a subgroup of 5 animals from each treated group, was anesthetized with halothane and killed by heart exsanguinations. Blood was collected *via* heart puncture with a heparized syringe and centrifuged at 2400 rpm for 15 min at 5°C to separate red blood cells from plasma. Plasma samples (0.5 ml) were each mixed with 0.5 ml autonitrite, then adjusted to pH 4.5 using 0.1 N acetic acid and processed for quantification of PB using the HPLC method that we developed and described above (54).

The total area under the PB concentration vs. time for plasma (AUC) was determined. The oral bioavailability was calculated as follows:

$$\text{Bioavailability} = \frac{\text{AUC}_{\text{po}}}{\text{AUC}_{\text{i.v}}} \times \frac{\text{Dose}_{\text{i.v}}}{\text{Dose}_{\text{po}}}$$

The oral bioavailability of PB was (mean \pm SE) $28.64 \pm 0.4\%$ when administered alone. On the other hand, concurrent exposure with dermal DEET or dermal permethrin significantly increased the oral bioavailability of PB to $51.04 \pm 2.3\%$ ($p < 0.01$) or $36.83 \pm 1.89\%$ ($p < 0.05$), respectively. Furthermore, combined administration with dermal DEET and dermal permethrin, resulted in an oral bioavailability of $46.90 \pm 2.94\%$ ($p < 0.01$) for PB.

b. Dermal bioavailability of DEET, Alone or When Applied concurrently with Oral PB and/or Dermal Permethrin

To determine the dermal bioavailability of DEET, two groups of 40 male Sprague-Dawley rats were given a single i.v dose of 40 mg/kg or 400 mg/kg DEET. To evaluate the dermal bioavailability of DEET with concurrent exposure to other chemicals, three groups of 40 rats were treated with a dermal dose of 400 mg/kg DEET, then one of these groups was treated with an oral dose of 13 mg/kg PB in water, a dermal dose of 1.3 mg/kg permethrin or a combination of both. Dermal application was carried out in ethanol, on an unprotected 1 cm² area of the pre-clipped skin on the back of the neck. A subgroup of 5 animals from each treatment group, was anesthetized with halothane and killed by heart exsanguinations. Blood was collected *via* heart puncture and DEET was quantified using our HPLC method described above (54).

The total area under the DEET concentration vs. time for plasma (AUC) was determined. The dermal (d) bioavailability was calculated as follows:

$$\text{Bioavailability} = \frac{\text{AUC}_d}{\text{AUC}_{i.v}} \times \frac{\text{Dose}_{i.v}}{\text{Dose}_d}$$

The dermal bioavailability of DEET was (mean \pm SE) 28.56 \pm 1.2% when administered alone. On the other hand, concurrent exposure to oral PB or dermal permethrin increased the dermal bioavailability of DEET to (mean \pm SE) 35.27 \pm 3.93% ($p < 0.01$) and 35.28 \pm 1.25% ($p < 0.01$), respectively. Also, combined administration with oral PB and dermal permethrin, resulted in a dermal bioavailability of 39.39 \pm 3.41% ($p < 0.01$) for DEET.

c. Dermal Bioavailability of Permethrin, Alone or When Applied Concurrently with Oral PB, and/or Dermal DEET

To determine the dermal bioavailability of permethrin, two groups of 40 male Sprague-Dawley rats were given a single i.v. or dermal dose of 1.3 mg/kg permethrin. To evaluate the dermal bioavailability of DEET with concurrent exposure to other chemicals, three groups of 40 rats were treated with a dermal dose of 1.3 mg/kg permethrin, then one of these groups was treated with an oral dose of 13 mg/kg PB in water, a dermal dose of 400 mg/kg DEET, or a combination of both. Dermal application was carried out in ethanol, on an unprotected 1 cm² area of the pre-clipped skin on the back of the neck. A subgroup of 5 animals from each treatment group, was anesthetized with halothane and killed by heart exsanguinations. Blood was collected *via* heart puncture and permethrin was quantified using our HPLC method described above (54).

The total area under the permethrin concentration vs. time for plasma (AUC) was determined. The dermal (d) bioavailability was calculated as follows:

$$\text{Bioavailability} = \frac{\text{AUC}_d}{\text{AUC}_{i.v}} \times \frac{\text{Dose}_{i.v}}{\text{Dose}_d}$$

The dermal bioavailability of permethrin was (mean \pm SE) 29.58 \pm 0.29% when applied alone. Concurrent exposure to oral PB greatly increased the dermal bioavailability of permethrin to (mean \pm SE) 116.20 \pm 0.33% ($p < 0.01$). On the other hand, dermal application of DEET resulted in a significant decrease in the dermal bioavailability of permethrin to (mean \pm SE) 22.43 \pm 0.35% ($p < 0.01$). Furthermore, combined administration with oral PB and dermal permethrin, resulted in a dermal bioavailability of (mean \pm SE) 45.12 \pm 0.5% ($p < 0.01$) for DEET.

Reportable Outcomes

Following are reportable outcomes from our studies:

1. Neurobehavioral deficits and blood-brain barrier and blood-testis barrier permeability following dermal application with DEET and permethrin, alone and in combination.
2. Locomotor and sensorimotor deficits following dermal application of DEET and permethrin and oral treatment with PB
3. Neuropathological changes in the central nervous system (CNS) of rats following sub-chronic dermal exposure with 1x doses of DEET (40 mg/kg) and permethrin (0.13mg/kg), alone and in combination. These changes are characterized by an increase in immunohistochemical reactivity with anti glialfibrillary acidic protein (GFAP), suggestive of extensive glial reactivity and irregular dendritic staining with anti microtubule-associated protein (MAP-2) that suggest abnormal dendritic branching following treatment with a combination of DEET and permethrin.
4. Blood-Brain Barrier (BBB) permeability and cholinergic changes following exposure to stress with a combination of pyridostigmine bromide (PB), DEET, and permethrin. Concurrent treatment with PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal) and permethrin (0.13mg/kg, dermal), alone or in combination with stress for 28 days resulted in a significant increase in the BBB permeability following treatment with combination of chemicals and stress. Cerebral cortex, white matter, deep gray matter and brainstem from the animals co-exposed with chemicals and stress exhibited significant HRP staining as compared with chemicals or stress alone. Brain regions showed a significant

inhibition in AChE activity and a decrease in m2 muscarinic acetylcholine receptor ligand binding densities following concurrent exposure with chemicals and stress.

5. Increased urinary excretion of 8-hydroxy-2'-deoxyguanosine following single dermal dose of DEET and permethrin, alone and in combination to rats. Exposure of rats to a single dose of DEET (400mg/kg, dermal in 70% ethanol) and permethrin (1.3 mg/kg, dermal in 70% ethanol), alone or in combination resulted in significantly higher excretion of 8-hydroxy-2'-deoxyguanosine in the animals exposed to DEET alone or in combination with permethrin than controls.

6. Urinary 3-nitrotyrosine excretion following combined exposure to DEET and permethrin by dermal application and oral administration of PB

7. Release of cytochrome c from brain mitochondria

8. Development of an analytical method to quantitate PB, DEET, and permethrin as well as their metabolites using HPLC.

9. Combined exposure to test compounds altered the bioavailability of single components. Thus, the oral bioavailability of PB was increased when combined with DEET or permethrin as well as following combined exposure to both DEET and permethrin. Also, dermal bioavailability of DEET was enhanced following combined exposure with oral PB and/or dermal permethrin. The bioavailability of dermal permethrin was greatly increased when administered with oral PB. On the other hand, combined exposure to dermal DEET decreased the bioavailability of dermal permethrin. Furthermore, the dermal bioavailability of permethrin was increased by combined application with oral PB and dermal DEET.

In summary, these data suggest that toxic interactions between DEET, permethrin and PB and consequent health effects are dependent on exposure to other compounding factors such as stress and any other environmental conditions.

Conclusions

The goal of the current project is to define the neurotoxic interactions between Pyridostigmine bromide (PB), DEET, and permethrin, and molecular mechanisms of neuropathological effects resulting as a consequence of co-exposure to these chemicals. Rats were exposed to doses that included levels enough to be representative of human exposure. In our pathological experiments rats were exposed to PB (1.3 mg/kg, in water oral), 40 mg/kg DEET and 1.3 mg/kg permethrin in 70% ethanol by dermal exposure for varying length of time. Additionally, the animals were subjected to restraining stress by placing them in Plexiglas cylinders for five minutes daily. These doses and routes of exposure were chosen to closely approximate the possible mode of exposure to these chemicals during the Gulf War.

The animals were evaluated for the breakdown of blood-brain barrier (BBB) by [³H]hexamethonium and horseradish peroxidase (HRP) uptake into the central nervous system. Gross histopathological evaluations of the CNS were carried out by H&E staining. Glial reactivity and neuronal dendritic loss were evaluated by immunohistochemical assessment with anti glialfibrillary acidic protein (GFAP) and anti microtubule-associated protein (MAP-2), respectively. CNS cholinergic system was studied by evaluating the acetylcholinesterase (AChE) activity and [³H]AFDX-384 ligand binding for m2 muscarinic acetylcholine receptor in brain regions. Furthermore, we evaluated the possibility of oxidative damage as a consequence of exposure to these chemicals, alone or in combination. To this end, we monitored urinary excretion of 8-hydroxy-2'-deoxyguanosine and 3-nitrotyrosine by HPLC, as a bio-marker of oxidative DNA damage and generation of oxygen free radicals, respectively. In addition, we also found that combined exposure to DEET and permethrin caused a significant increase in brain mitochondrial release of cytochrome c.

The data from these studies show that:

1. Daily dermal exposure to DEET and permethrin, alone or in combination for 60 days caused a significant neuronal damage as evaluated by H&E staining.
2. Daily dermal exposure to DEET and permethrin, alone or in combination for 60 days caused a significant increase in the expression of GFAP.
3. Following treatment with DEET and permethrin alone, and in combination for 60 days, MAP-2 expression was significantly decreased in cerebellum and cortex.
4. A significant increase in the BBB permeability was observed following concurrent treatment with PB, DEET, and permethrin combined with stress for 28 days as assessed by [³H]hexamethonium iodide uptake.
5. Cerebral cortex, white matter, deep gray matter and brainstem from the animals co-exposed with PB, DEET, and permethrin and stress for 28 days exhibited significant HRP staining as compared with chemicals or stress alone.
6. Brain regions showed a significant inhibition in AChE activity and a decrease in m2 muscarinic acetylcholine receptor ligand binding densities following co-exposure with DEET, permethrin and PB and stress for 28 days.
7. There were a significantly higher levels of urinary excretion of 8-hydroxy-2'-deoxyguanosine in the animals exposed to DEET alone or in combination with permethrin urine than the controls.
8. Permethrin exposure alone caused an increase in the levels of 8-hydroxy-2'-deoxyguanosine in the urine, but these values were statistically not significant.

9. The oral bioavailability of PB was increased following combined exposure with DEET and/or permethrin. Similarly, the dermal bioavailability of DEET, was enhanced after concurrent administrations with oral PB and/or dermal permethrin. Also, although the dermal bioavailability of permethrin was decreased when applied with dermal DEET, it was enhanced following simultaneous administrations of oral PB and/or dermal DEET. We would like emphasize here that these bioavailability studies were carried out following acute exposure to these chemicals, alone or in combination, whether a similar pattern of bioavailability and pharmacokinetics exists following chronic exposure, remains to be seen. Further studies are required to answer these questions.

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Appendix

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1. Abu-Qare, A.W., and Abou-Donia, M.B (2000). Simultaneous determination of pyridostigmine bromide, *N,N*-diethyl-*m*-toluamide, permethrin, and their metabolites in rat plasma and urine by high-performance liquid chromatography. *J. Chromatography*, **749**: 171-178.
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1. Goldstein, L.B., Dechkovskaia, A., Bullman, S., Jones, K.H., Herrick, E.A., Abdel-Rahman, A., Khan, W.A., and Abou-Donia, M.B.(2001).Daily dermal co-exposure of rats to DEET and permethrin, produces sensorimotor deficit and changes in blood-brain barrier (BBB) and blood-testis barrier (BTB). *The Toxicologist.* **60**: 59
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Simultaneous determination of pyridostigmine bromide, *N,N*-diethyl-*m*-toluamide, permethrin, and their metabolites in rat plasma and urine by high-performance liquid chromatography

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Abstract

A rapid and simple method was developed for the separation and quantification of the anti nerve agent drug pyridostigmine bromide (PB; 3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide) its metabolite *N*-methyl-3-hydroxypyridinium bromide, the insect repellent DEET (*N,N*-diethyl-*m*-toluamide), its metabolites *m*-toluamide and *m*-toluic acid, the insecticide permethrin (3-(2,2-dichloro-ethenyl)-2,2-dimethylcyclopropanecarboxylic acid(3-phenoxyphenyl)methyl ester), and two of its metabolites *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid in rat plasma and urine. The method is based on using C₁₈ Sep-Pak® cartridges for solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) with reversed-phase C₁₈ column, and gradient UV detection ranging between 208 and 230 nm. The compounds were separated using gradient of 1 to 99% acetonitrile in water (pH 3.20) at a flow-rate ranging between 0.5 and 1.7 ml/min in a period of 17 min. The retention times ranged from 5.7 to 14.5 min. The limits of detection were ranged between 20 and 100 ng/ml, while limits of quantitation were 150–200 ng/ml. Average percentage recovery of five spiked plasma samples were 51.4±10.6, 71.1±11.0, 82.3±6.7, 60.4±11.8, 63.6±10.1, 69.3±8.5, 68.3±12.0, 82.6±8.1, and from urine 55.9±9.8, 60.3±7.4, 77.9±9.1, 61.7±13.5, 68.6±8.9, 62.0±9.5, 72.9±9.1, and 72.1±8.0, for pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid, respectively. The relationship between peak areas and concentration was linear over the range between 100 and 5000 ng/ml. This method was applied to analyze the above chemicals and metabolites following their administration in rats. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Pyridostigmine bromide; *N,N*-Diethyl-*m*-toluamide; Permethrin

1. Introduction

Simultaneous exposure to pyridostigmine bromide, DEET and permethrin has resulted in enhanced of neurotoxicity in hens [1], and caused significant

increase in lethality in rats [2]. Also acute interaction resulted in seizures and death following combined application of pyridostigmine bromide and DEET in mice [5]. Based on these reports, combined chemical exposure has been proposed as a possible cause of Gulf War veterans illness [1,3–8]. Pyridostigmine bromide was used as an antidotal drug against possible attack by organophosphate nerve agents, DEET was applied as insect repellent on the skin of

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veterans, and permethrin was sprayed against orthopod vectors on the battle dress uniforms [35]. Pyridostigmine bromide has been reported to be absorbed into plasma and excreted in urine following oral or intravenous dose in rat [9,10,17,18,27,29,36], in man [16,24], and in dog [15,28]. Absorption and excretion of DEET and metabolites were rapid after dermal application in human [11,13], in rats [19], and in dogs [14]. Permethrin was also reported to be absorbed into plasma, metabolized and excreted as metabolites in the urine following oral or intravenous dose in rats [20], and in rabbits [23].

Several analytical methods have been used for identification and quantification of the above chemicals and their metabolites, when applied alone in plasma and urine samples. These methods used high-performance liquid chromatography (HPLC) [9,11–14,25,34], HPLC–mass spectrometry [19], gas chromatography [21–24,39–41], gas chromatography–mass spectrometry [26,32,38,42], and thin layer chromatography [22,30,31]. Other techniques were also used, e.g. Micellar electrokinetic chromatography (MEKC) [33], radiochromatoelectrophoresis [34], electrophoresis with paper chromatography [36], and radioaminoassay [36]. Limits of detection of the chemicals and metabolites in plasma or urine samples when analyzed using HPLC–UV, following individual application were ranged between 10 and 100 ng/ml [11,20,25], while their recoveries were between 65 and 95% [20,25,37].

In this study we present a reliable method for simultaneous analysis of the above chemicals and their metabolites in rat plasma and urine using solid-phase extraction (SPE) coupled with reversed-phase HPLC (RP-HPLC).

2. Experimental

2.1. Chemicals and materials

DEET (*N,N*-Diethyl-*m*-toluamide) (Fig. 1) was obtained from Aldrich Chem Co., Inc. (Milwaukee, WI, USA), Pyridostigmine bromide (PB; 3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide), *m*-phenoxybenzoic acid, and *m*-phenoxybenzyl alcohol (Fig. 1) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Permethrin(3-

(2,2-dichloro-ethenyl)-2,2-dimethylcyclopropanecarboxylic acid(3-phenoxyphenyl) methylester) was obtained from Chem Service, Inc. (West Chester, PA, USA), *m*-Toluamide, and *m*-toluic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA), *N*-methyl-3-hydroxypyridinium bromide was prepared following the method by Somani et al. [16]. Water (HPLC grade) and acetonitrile were obtained from Mallinckrodt Baker, Inc. (Paris, Kentucky, USA). C₁₈ Sep-Pak^R cartridges were obtained from Waters Corporation (Waters Corporation, Milford, MA, USA).

2.2. Animals

Rats (Sprague–Dawley) were purchased from Zivic Miller (Zelienople, PA, USA). The animals were kept in plastic metabolic cages. Three groups, each of five rats were treated with a single oral dose of 13 mg/kg of pyridostigmine bromide, a single dermal dose 400 mg/kg of DEET, and a single dermal dose of 1.3 mg/kg of permethrin. Another group of five rats were treated with a combination of the above chemicals. Five untreated control rats were treated with oral dose of water, or dermal dose of ethanol. The animals were held in metabolic cages allow collection of urine samples. Urine samples were collected from treated and control rats after 8 h of dosing. The animals were anesthetized with halothane and scarified by heart exsanguinations at 8 h. Blood was collected via heart puncture with a heparinized syringe and centrifuged at 2400 rev./min for 15 min at 5°C to separate plasma. Urine and plasma samples were stored at –20°C prior to analysis.

2.3. Instrumentation

The liquid chromatographic system (Waters 2690 Separation Module), consisted of a Waters 600E Multisolvant delivery system pumps, a Waters Ultra WISP 715 autoinjector, and a Waters 2487 Dual λ absorbance detector (Waters Corporation, Milford, MA). A guard column (Supelco, 2 cm \times 4.0 mm, 5 μ m (Supelco Park, Bellefonte, PA), and a reversed-phase C₁₈ column μ BondapakTM C₁₈ 125A $^{\circ}$ 10 μ m, 3.9 \times 300 mm were used, (Waters Corporation, Milford, MA).

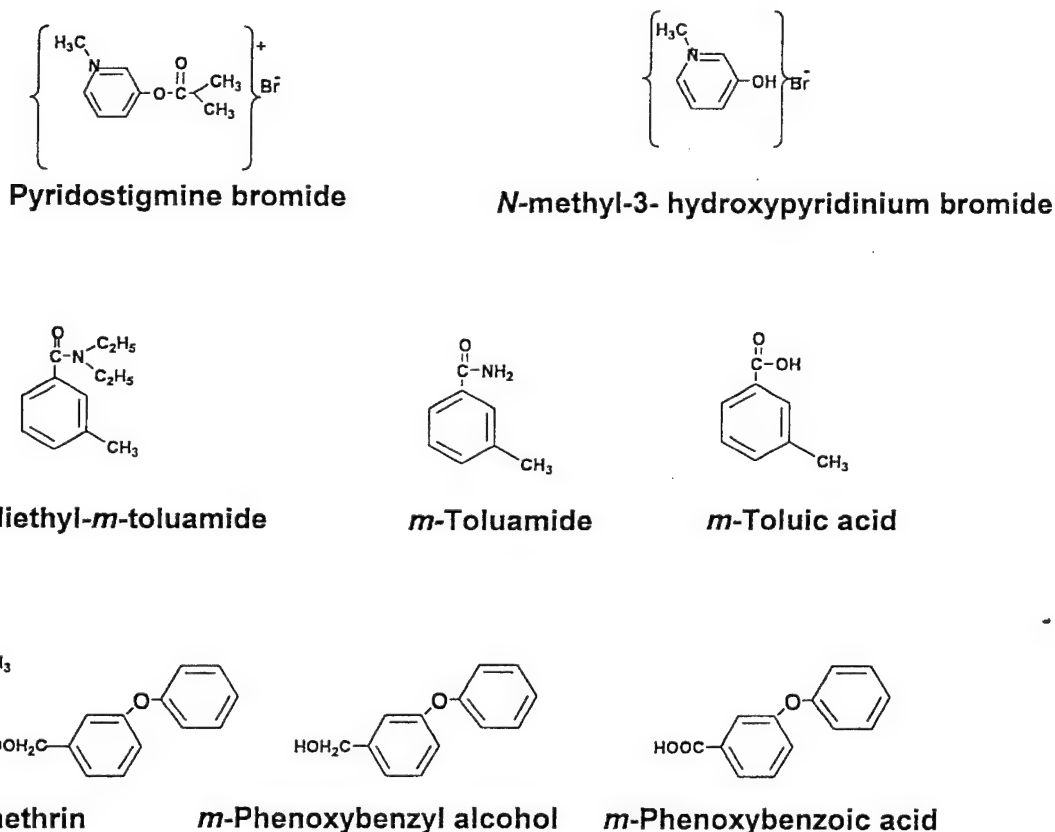


Fig. 1. Chemical structures of pyridostigmine bromide, DEET, permethrin, N-methyl-3-hydroxypyridinium bromide, m-toluamide, m-toluic acid, m-phenoxybenzyl alcohol, and m-phenoxybenzoic acid.

2.4. Sample preparation

A 0.2 ml plasma and urine samples from untreated rats were spiked with concentrations ranging between 100 and 5000 ng/ml of each of pyridostigmine bromide, DEET, permethrin, N-methyl-3-hydroxypyridinium bromide, m-toluamide, m-toluic acid, m-phenoxybenzoic acid, and m-phenoxybenzyl alcohol. Spiked and treated samples were acidified with 1 N acetic acid (pH 5.0). Disposable C₁₈ Sep-Pak Vac 3cc (500 mg) cartridges (Waters Corporation, Milford, MA) were conditioned with 3 ml of acetonitrile, then equilibrated using 3 ml of water prior to use. The spiked urine and plasma samples were vortexed for 30 s, centrifuged for 5 min at 1000 g, and the supernatant was loaded into the disposable cartridges, then washed with 2 ml of water, and eluted two times by 1 ml of methanol, then twice by

1 ml of acetonitrile, and reduced to 500 μ l using a stream of nitrogen, prior to analysis by HPLC.

2.5. Chromatographic conditions

A 10 μ l solution of plasma or urine residues was injected into HPLC. The mobile phase was water (adjusted to pH 3.20 using 1 N acetic acid):acetonitrile gradient at flow-rate programmed from 0.5 to 1.7 ml/min. The gradient started at 1% acetonitrile, increased to 75% acetonitrile at 6 min, then increased to 99% acetonitrile by 11 min. Then the system returned to 1% acetonitrile at 15 min where it was kept under this condition for 2 min to re-equilibrate. The eluents were monitored by UV detection of wavelength of 208 nm for pyridostigmine bromide and N-methyl-3-hydroxypyridinium bromide, 210 nm for DEET, m-toluamide, and m-

toluic acid and at 230 nm for permethrin, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid. The chromatographic analysis was performed at ambient temperature.

2.6. Calibration procedures

Five different calibration standards of a mixture of pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid were prepared in acetonitrile. Their concentrations ranged from 100 to 5000 ng/ml. Linear calibration curves were obtained by plotting the peak areas of the individual chemicals as a function of the concentration using GraphPad Prism program for windows (GraphPad Software, Inc., San Diego, CA, USA). The standard curves were used to determine recovery of the chemicals from plasma and urine samples.

2.7. Limits of detection and limits of quantitation

Limits of detection (LOD) were determined at the lowest concentration to be detected, taking into consideration a 1:3 baseline noise: calibration point ratio. A reproducible lowest possible concentration was considered as the limit of quantitation (LOQ). The LOQ was repeated five times for confirmation.

3. Results

3.1. Standard calibration curves

The standard calibration curves of peak area against concentration of pyridostigmine bromide, DEET, and permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid are shown in Fig. 2. Linearity of the calibration curves for the three compounds was achieved at concentrations ranging from 100 to 5000 ng/ml.

3.2. Chromatogram

Chromatographic profiles were obtained for rat plasma and urine samples after solid-phase extraction

using C₁₈ Sep Pak[®] cartridges under HPLC conditions, described above (Figs. 3 and 4). Retention times were 6.8, 9.5, 14.4, 5.7, 7.5, 8.6, 10.7 and 11.3 min for pyridostigmine bromide, DEET, and permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, respectively. The total run time was 17 min. Clean chromatogram shows no interference from endogenous substances in plasma and urine samples. This suggests an efficient sample preparation and clean up method.

3.3. Extraction efficiency and recovery

The average extraction recoveries of pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzoic acid, and *m*-phenoxybenzyl alcohol were determined at concentrations ranged between 100 and 5000 µg/ml (Tables 1 and 2).

Spiked plasma and urine samples were extracted and analyzed for each concentration in five replicates. Average percentage recoveries were 51.4±10.6, 71.1±11.2, 82.3±6.7, 60.4±11.8, 63.6±10.1, 69.3±8.5, 68.3±12.0 and 82.6±8.1 from plasma, and 55.9±9.8, 60.3±7.4, 77.9±9.1, 53.9±9.7, 64.2±6.5, 71.7±4.2, 86.5±6.1 and 89.7±4.1 from urine for pyridostigmine bromide, DEET, and permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, respectively.

3.4. LOD

Blank plasma and urine samples from untreated rats were used as references for plasma and urine collections. LOD were calculated from a peak signal-to-noise ratio of 3:1. The resulting detection limits range were 100, 50, 50, 100, 100, 80, 20 and 30 for pyridostigmine, DEET, permethrin, *N*-methyl-3-hydroxypyridine, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzylalcohol, and *m*-phenoxy benzoic acid, respectively.

3.5. LOQ

LOQ were determined to be 150 ng/ml for pyridostigmine bromide and DEET and 100 ng/ml

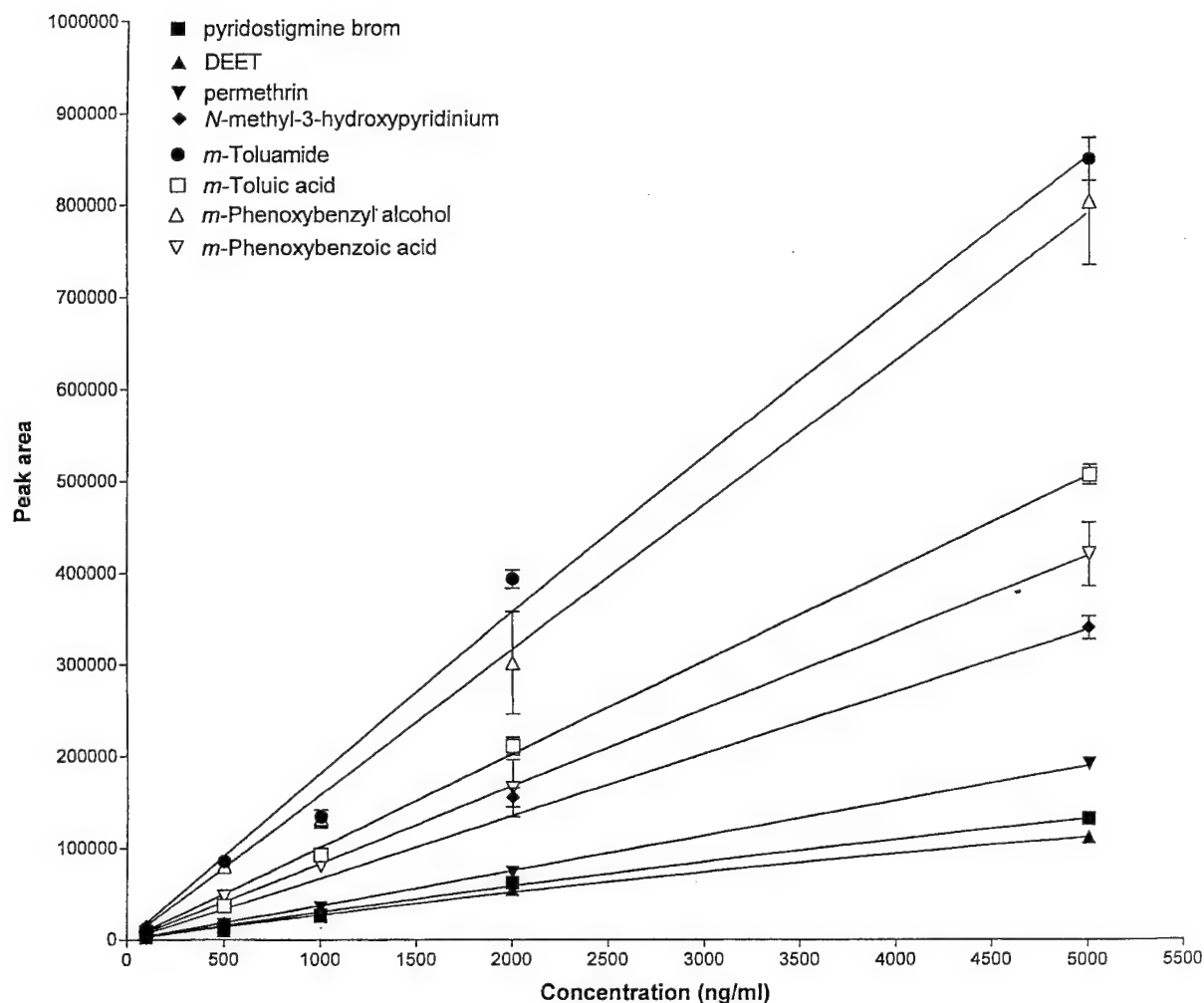


Fig. 2. Standard calibration curves of pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid.

for permethrin in plasma. In urine limits of quantitation were 200, 150, and 100, 150, 150, 100, 100, 100 ng/ml for pyridostigmine bromide, DEET, and permethrin, *N*-methyl-3-hydroxypyridine, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, respectively.

3.6. Application of the method to biological samples

In order to validate the method, the method was applied for analysis of the chemicals in treated rats, when applied alone or in combination. The rats were

sacrificed at 8 h following dosing. In plasma, their levels were 224 ± 123 , 1320 ± 346 and 182 ± 76 ng/ml for pyridostigmine bromide, DEET, and permethrin, while concentration of metabolites in plasma were 107.3 ± 21.5 , 98.4 ± 14.8 , 107.5 ± 5.7 and 142.7 ± 27.1 ng/ml for *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, respectively. *N*-methyl-3-hydroxypyridinium bromide a metabolite of pyridostigmine bromide was not detected in rat plasma. Levels of pyridostigmine bromide and DEET in rat urine were 712 ± 186 ng/ml and 3.2 ± 0.82 μ g/ml, respectively. DEET metabolites *m*-toluamide and *m*-toluic acid, and

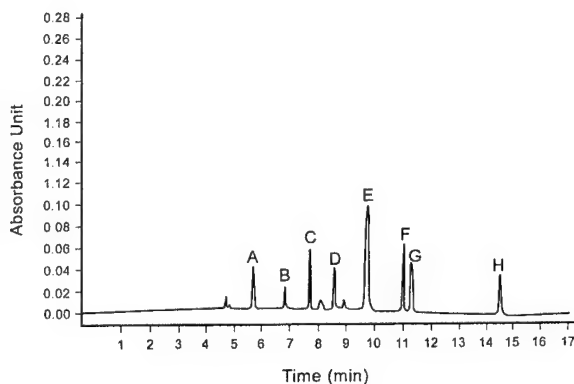


Fig. 3. Chromatogram of plasma sample of (A) *N*-methyl-3-hydroxypyridinium bromide; (B) PB (pyridostigmine bromide); (C) *m*-toluamide; (D) *m*-toluic acid; (E) DEET; (F) *m*-phenox-ybenzyl alcohol; (G) *m*-phenoxybenzoic acid; and (H) permethrin under established HPLC conditions.

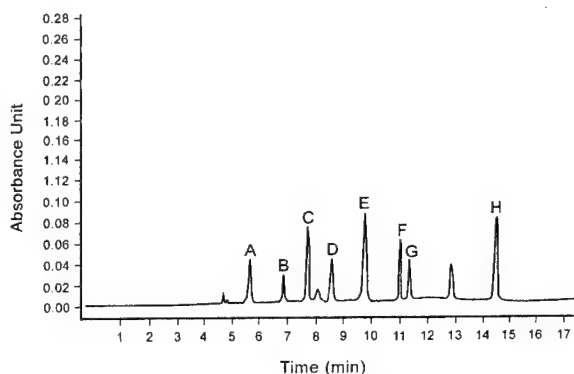


Fig. 4. Chromatogram of spiked urine sample with (A) *N*-methyl-3-hydroxypyridinium bromide; (B) PB (pyridostigmine bromide); (C) *m*-toluamide; (D) *m*-toluic acid; (E) DEET; (F) *m*-phenox-ybenzyl alcohol; (G) *m*-phenoxybenzoic acid; and (H) permethrin under established HPLC conditions.

permethrin and its metabolites *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid, have not been detected in urine after 8 h of dosing.

4. Discussion

The present study reports the development of an HPLC method for quantitative and qualitative analysis of pyridostigmine bromide, DEET, permethrin and their metabolites in plasma and urine of treated rats.

Linearity of standard calibration curves for the chemicals in the present method is in consistent with previous reports. Eilln et al. [9] reported linear range between 40 and 500 ng/ml for DEET in plasma using HPLC, while Yaylor et al. [14] reported a linearity over a range between 19 and 1910 ng/ml for DEET using gas chromatography (GC). Also, Chan et al. [38] reported a linear range for pyridostigmine bromide in human plasma over concentrations between 50 and 1000 ng/ml.

The chromatogram obtained following SPE and HPLC analysis shows no interference from plasma and urine subjects, indicating an efficient clean up method used. Also simultaneous and rapid analyses of the parent compounds and metabolites are cost efficient and save time for sample preparation.

Recoveries of the chemicals and metabolites were suitable for application of the method for analysis of treated samples for parent compounds and their metabolites. Low recovery for pyridostigmine bromide might have resulted from the use of solvent system that was not quite suitable for extracting pyridostigmine bromide, and at the same time it was needed for extracting and analyzing the other two

Table 1
Percentage recovery of pyridostigmine bromide, DEET permethrin, and metabolites in rat plasma^a

Concentration (ng/ml)	Pyridostigmine bromide	DEET	Permethrin	<i>N</i> -methyl-3-hydroxypyridinium bromide	<i>m</i> -Toluic acid	<i>m</i> -Toluamide	<i>m</i> -Phenox-ybenzyl alcohol	<i>m</i> -Phenox-ybenzoic acid
5000	61.8±5.4	81.4±4.3	81.4±7.6	53.4±13.6	61.5±12.1	73.4±13.6	65.7±18.2	89.2±9.7
2000	63.8±7.1	84.1±9.5	87.1±9.3	64.7±18.3	65.3±7.2	64.7±9.8	61.5±13.8	80.1±10.2
1000	55.5±10.7	73.6±8.7	82.1±8.3	57.1±9.2	68.4±17.6	70.2±4.1	68.7±10.2	73.6±8.3
200	58.4±13.6	71.2±4.8	72.1±6.7	59.8±7.4	60.5±13.8	71.8±8.6	75.3±8.2	86.7±6.4
100	44.1±10.9	60.7±16.7	71.5±8.9	66.2±10.4	62.1±4.6	66.2±6.5	70.4±9.7	83.4±5.8

^a Values are expressed as mean±SD of five replicates.

Table 2

Percentage recovery of pyridostigmine bromide, DEET permethrin, and metabolites from rat urine^a

Concentration (ng/ml)	Pyridostigmine bromide	DEET	Permethrin	<i>N</i> -methyl-3-hydroxypyridinium bromide	<i>m</i> -Toluamide	<i>m</i> -Toluic acid	<i>m</i> -Phenoxybenzyl alcohol	<i>m</i> -Phenoxybenzoic acid
5000	54.1±7.3	62.8±10.9	90.7±3.9	62.2±12.8	69.2±10.6	59.8±12.3	66.8±6.5	75.6±4.1
2000	53.8±8.4	58.1±7.5	83.1±4.2	52.7±10.6	61.9±9.7	63.4±8.7	73.8±12.3	71.9±12.1
1000	49.6±8.3	52.7±7.4	70.8±8.7	61.8±9.3	75.3±7.6	69.7±5.2	82.6±7.8	77.8±3.6
200	48.0±13.2	60.9±8.2	71.7±10.1	64.7±8.4	71.9±5.6	61.2±14.7	71.9±5.8	65.4±9.5
100	52.3±8.5	57.6±10.7	68.4±4.0	67.2±13.2	64.5±11.2	58.4±6.5	69.8±13.1	69.8±10.9

^a Values are expressed as mean±SD of five replicates.

chemicals and metabolites under similar conditions. Also hydrolysis of pyridostigmine bromide during the extraction is possible, in a previous study Aquilonius and Hartvig [43] reported that extraction and analysis of pyridostigmine bromide was a challenge to the analytical chemists, because of its *in vitro* hydrolysis could take place in buffer solutions, plasma and blood. Percentage recoveries depends on the matrix, extracting solvent, method of analysis, and the amount to be analyzed. Recoveries of DEET from serum and urine were reported to be 93–95%, and 65–70%, respectively using GC–MS as an analytical technique [38], while recovery of DEET from water samples was 45.6% using Micellar kinetic chromatography method [33]. Hennis et al. [15] reported a recovery of 50% of *N*-methyl-3-hydroxypyridinium from dog plasma and urine, while Chan et al. [37] reported a recovery of 82% of pyridostigmine bromide from plasma at low concentration of 50 ng/ml, while its recovery was 92% when a concentration of 400 ng/ml was used. In previous studies, recovery of pyrethroids and metabolites from rat urine ranged between 90 and 98% using GC–MS [26], while the recovery was 92% at high concentration of 400 ng/ml using GC. In our method, recoveries differed with individual chemicals. Recoveries of the chemicals analyzed in our method was between 55 and 83%. This range lies within the reported values in the literature, taking into consideration simultaneous analysis of the parent chemicals and their metabolites.

The LOD reported in our method allow to analyzing samples from treated animals following doses resemble real exposure. Our ability to detect the three compounds and metabolites in plasma after 8 h of dosing is an evidence of the method suitability.

LOD and LOQ depended upon the nature of the matrix, rate of application, and method of analysis [10,38–40]. Pyridostigmine bromide and DEET were also detected in urine samples at 8 h, while failure to detect permethrin and metabolites in urine might be due to the low dermal dose of permethrin that used (1.3 mg/kg), its low absorption through skin, and to rapid hydrolysis and conjugation of permethrin and the targeted metabolites. Hennis et al. [15] reported a 50 ng/ml as a limit of detection of *N*-methyl-3-hydroxypyridinium in dog plasma using ion-exchange liquid chromatography, while Miller and Verma [36] reported a 2.5 ng/ml as detection limit of pyridostigmine bromide in tissues using radioimmunoassay method, while using HPLC technique, limits of detection of pyridostigmine bromide in plasma was 10 ng/ml [25], and ranged between 2.7 and 18.6 ng/ml in plasma using GC [41]. The detection limit of DEET was 90 and 90 ng/g from urine and serum, respectively, using HPLC–UV method [11], and 15 ng/ml for DEET in human and dog plasma using HPLC [34], while it was 25 ng in cosmetic products using high-performance thin-layer chromatography (HPTLC) method [30]. Detection limits of permethrin in urine samples were 0.3–0.5 µg/l using GC–MS technique [26], and 5 µg/l in plasma using GC method [39]. The reported LOD in the literature are consistent with our results for the simultaneous analysis of the combined chemicals and their metabolites, which ranged between 20 and 100 ng/ml.

A rapid and simple HPLC method was developed for separation and residual determination of pyridostigmine bromide, DEET, permethrin and selected metabolites in rat spiked and treated plasma and urine samples. SPE was used which selectively

extracted the above chemicals from plasma and urine samples without interference of an expected mixture of metabolites and endogenous compounds. The method could be applied routinely for monitoring of the above chemicals in human plasma and urine samples of persons exposed to the combined chemicals. This method could also be used in pharmacokinetics studies to assess distribution of the parent compounds and metabolites in body tissues and fluids. The use of SPE is advantageous compared to liquid–liquid extraction which is a time consuming and requires large amounts of organic solvents. The main advantage of the method is the ability to analyze simultaneously the three chemicals and their metabolites under similar conditions, saving time and expenses for sample preparation.

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Increased 8-hydroxy-2'-deoxyguanosine, a biomarker of oxidative DNA damage in rat urine following a single dermal dose of DEET (*N,N*-diethyl-*m*-toluamide), and permethrin, alone and in combination

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Abstract

Levels of the biomarker of DNA oxidative damage 8-hydroxy-2'-deoxyguanosine (8-OHdG) in rat urine following dermal exposure to DEET (*N,N*-diethyl-*m*-toluamide) and permethrin, alone and in combination have been determined. A group of five rats for each time point were treated with a single dermal dose of 400 mg/kg of DEET, 1.3 mg/kg of permethrin or their combination. Urine samples were collected 2, 4, 8, 16, 24, 48, and 72 h following application. Control urine samples of rats treated with ethanol were also collected at the same time intervals. Solid phase extraction coupled with high performance liquid chromatography (HPLC) with UV detection at 254 nm was used for determination of 2'-deoxyguanosine, and (8-OHdG). The limits of detection (LOD) were 0.5 ng of both 2'-deoxyguanosine and 8-OHdG. Their average percentage recoveries from urine samples were between 70–85%. A single dermal dose of DEET or in combination with permethrin significantly induced levels of (8-OHdG) that are excreted in the urine over the time course of the study compared to control urine samples. Permethrin did not cause significant increase in the amount of 8-OHdG in the urine. Levels of 8-OHdG in urine excreted at 24 h were 1009 ± 342 , 1701 ± 321 , 1140 ± 316 , and 1897 ± 231 ng following treatment with ethanol, DEET, permethrin, and DEET + permethrin, respectively. The results indicate that dermal administration of DEET could generate free radical species hence cause DNA oxidative damage in rats. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *N,N*-diethyl-*m*-toluamide; Permethrin; DNA oxidative damage; Free radicals

1. Introduction

Oxidative DNA damage is emerging as a biomarker of effect in studies assessing the health risks of occupational chemicals. DNA damage can lead to mutation, which can be reflected in more severe biological consequences such as genetic

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disease and cancer (Joseph et al., 1997). Exogenous and endogenous oxidants frequently cause oxidative damage to DNA (Malins, 1993; Halliwell, 1999). Because the reactive oxidants are not suitable for analysis, oxidized bases like 8-hydroxy-2'-deoxyguanosine (8-OHdG) are used as biomarkers for DNA oxidative damage (Halliwell and Dizdaroglu, 1992; Loft et al., 1993; Kasai, 1997; Helbock et al., 1999; Anson et al., 2000). Determination level of (8-OHdG) in urine and tissues following exposure to environmental toxicants has been reported, e.g. *p*-dichlorobenzene (Umemura et al., 2000), trichloroethylene and perchloroethylene (Toraason et al., 1999), safrole (Liu et al., 1999), 2-nitropropane (Loft et al., 1998a) in rats, δ -aminolevulinic acid in Chinese hamster ovary (CHO) cells (Yusof et al., 1999), and dieldrin in fish (Rodriguez-Ariza et al., 1999). Also 8-OHdG was produced as a result of high fat diet in rats (Loft et al., 1998b), in tobacco smokers (Howard et al., 1998), after exposure to copper in rats (Toyokuni and Sagripanti, 1994), and following exposure to *m*-phenylenediamine and its derivative in the presence of copper in humans (Chen et al., 1998), after acute iron intoxication in rat sperm cells in vivo and in vitro (Wellejus et al., 2000), and following incubation of Chinese hamster cells with metabolites of *o*-phenylphenol (Henschke et al., 2000). Furthermore, an increased in the urinary levels of (8-OHdG) was considered as a biomarker of oxidative stress in Down Syndrome in human subjects (Jovanovic et al., 1998), and its level was correlated with increased age in experimental animals (Drury et al., 1998; Mecocci et al., 1999; Lodovici et al., 2000), also to DNA damage in cases of Alzheimer's disease (Nunomura et al., 1999), and after nucleosides were photosensitized with dyes (Kvam et al., 1994). Also it was used as a biomarker following exposure to X and γ -rays in mice (Kasai et al., 1986).

High performance liquid chromatography (HPLC) with electrochemical detection has been used for measurement concentration of (8-OHdG) in urine (Ravanat et al., 1995; Cooke et al., 1998; Jovanovic et al., 1998; Bogdanov et al., 1999), in tissues (Floyd et al., 1986; Wise-

man et al., 1995; Gedik et al., 1998; Cadet et al., 1998; Shen et al., 1999; Toraason et al., 1999; Mecocci et al., 1999; Takeuchi et al., 1999; Umemura et al., 2000), and in serum (Cooke et al., 1998). Other methods have also been used, such as gas chromatography-mass spectrometry (Wiseman et al., 1995; England et al., 1998; Evans et al., 1999; Ravanat et al., 1999); Comet assay (Gedik et al., 1998; Piperakis et al., 1999), and polyclonal antibodies (Degan et al., 1991). Recently, a method using HPLC-mass spectrometry has been developed for analysis of 8-OHdG in urine samples (Renner et al., 2000).

Permethrin is a pyrethroide insecticide effective against head lice (Burgess et al., 1992), and against lyme disease (Miller, 1989). DEET is applied as an insect repellent (Brown and Hebert, 1997). Both chemicals were used by US military personnel during the Persian Gulf War to protect veterans against mosquitoes and biting insects (Young and Evans, 1998).

Permethrin modifies sodium channel to open longer during a depolarization pulse (Narahashi, 1985). Previous studies showed that DEET has direct effect on the nervous system in laboratory animals resulting in spongiform myelinopathy in the brain stem with signs include ataxia, seizures, coma and death (Verschoyle et al., 1990). Furthermore, extensive and repeated topical application of DEET resulted in human poisoning including two deaths (Roland et al., 1985; Edwards and Johnson, 1987). Combined exposure to permethrin and DEET enhanced neurotoxicity of individual chemicals in hens (Abou-Donia et al., 1996), and increased mortality in rats (McCain et al., 1997). Published reports implicated exposure to DEET and permethrin with Gulf War Illnesses (Abou-Donia et al., 1996; Haley and Kurt, 1997; Kurt, 1998; Shen, 1998; Wilson et al., 1998; Hoy et al., 2000).

No published reports examined possible effect of DEET and permethrin, alone or in combination on oxidative DNA damage. In this study, we present results of analysis of urinary levels of (8-OHdG), as a biomarker of DNA oxidative damage following a dermal dose of DEET and permethrin in rats.

2. Materials and methods

2.1. Chemicals and materials

8-Hydroxy-2'-deoxyguanosine and 2'-deoxyguanosine (Fig. 1) were obtained from Sigma Co Inc., (St. Louis, MO). DEET ($\geq 97\%$, *N,N*-diethyl-*m*-toluamide) was purchased from Aldrich Chemical Co, Inc. (Milwaukee, WIS), while permethrin (99%, 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl)methyl ester) was obtained from Chem Service, Inc. (West Chester, PA). Waters (HPLC grade) and acetonitrile were obtained from Mallinckrodt Baker, Inc. (Paris, Kentucky). C_{18} Sep-Pak^R Cartridges were obtained from Waters Corporation (Milford, MA).

2.2. HPLC system

The liquid chromatographic system (Waters 2690 Separation Module), consisted of a Waters 600E Multisolvant delivery system pumps, a Waters Ultra WISP 715 autoinjector and a Waters 2487 Dual λ absorbance detector (Waters Corporation, Milford, MA). A guard column (Supelco, 2 cm \times 4.0 mm, 5 μ m, Supelco Park, Bellefonte, PA), and a reversed-phase C_{18} column μ BondapakTM C_{18} 125 Å 10 μ m, 3.9 \times

300 mm were used, (Waters Corporation, Milford, MA).

2.3. Calibration curve, recovery and limits of detection

Standard calibration curve of a concentration between 1–10 ng of 2'-deoxyguanosine, and 8-OHdG were obtained under the described HPLC conditions. Their detection limits were determined as the lowest concentration that can be detected taking into consideration a 1:3 base line: peak signal ratio.

Recoveries of the chemicals from urine samples were determined for concentrations of 1–10 ng. A known concentration was spiked with control urine samples (previously analyzed) and the samples were re-analyzed as described under sample preparation. Amounts of 2'-deoxyguanosine and 8-OHdG were corrected based on the recovery obtained.

2.4. Experimental animals

Sprague-Dawley rats (200–240 g) were purchased from Zivic Miller (Zelienople, PA). The untreated animals were kept in a 12 h light/dark cycle (temperature 21–23°C) and were provided with a free supply of feed (Rodent Laboratory Chow, Ralston Purine Co., St. Louis, MO) and tap water. Animal care was conducted according to institutional guidelines.

2.5. Animals treatment

Permethrin and DEET were dissolved in 70% ethanol. A single dose of 1.3 mg/kg of permethrin and a single dose of 400 mg/kg of DEET were applied with a micropipette to an unprotected 1 cm² area of pre-clipped skin on the back of each rat. A group of five animals was used for each time point. Combined single dermal dose of 1.3 mg/kg of permethrin, followed by a single dermal dose of 400 mg of DEET was also applied. Five control rats were treated with equal volume of 70% ethanol and kept under similar conditions as treated rats.

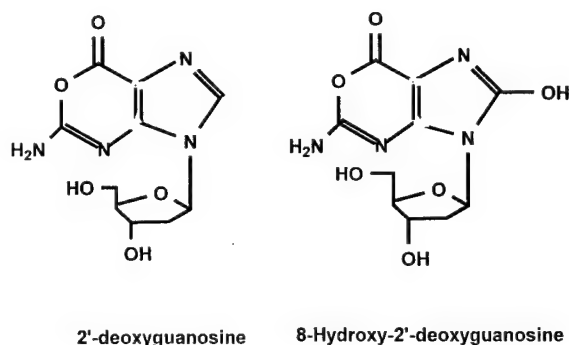


Fig. 1. Structures of 2'-deoxyguanosine to 8-hydroxy-2'-deoxyguanosine.

2.6. Animals handling

After dosing, each rat was placed in a metabolic cage, and urine samples were collected at 2, 4, 8, 16, 24, 48, and 72 h after dosing. After each time point the animals were sacrificed. A 2 ml of the collected volume at each time point was taken for analysis. The samples were frozen at -20°C until analysis.

2.7. Sample preparation

A volume of 2.0 ml of the urine samples was acidified (pH 5.00) using 0.1 N acetic acid, then applied on a disposable C_{18} Sep-Pak Vac 3cc (500 mg) cartridges (Waters Corporation, Milford, MA) previously conditioned with 2 ml of methanol, and equilibrated using 2 ml of water and 2 ml of 0.03 M phosphate dibasic buffer (pH 5) prior to use. After washing with 2 ml of potassium phosphate dibasic buffer (pH 5) and 2 ml of water, the sample was eluted using 2×1 ml of methanol, the methanolic eluates were reduced to 500 μl using stream of nitrogen, prior to analysis by HPLC.

2.8. Analysis

A volume of 10.0 μl of the extracts was injected into the HPLC system as described above. The mobile phase consisted of 83% water (adjusted to pH 3.00 using 0.1 M acetic acid), and 17% acetonitrile at flow rate of 0.50 ml/min. The eluents were monitored by UV detection at 254 nm. The chromatographic analysis was performed at ambient temperature. Amount of 8-Hydroxy-2'-deoxyguanosine and 2'-deoxyguanosine was calculated and corrected for the urine volume and total body weight of the animal.

2.9. Statistical analysis

Analysis of variance using GraphPad Prism program for Windows (GraphPad Software, Inc., San Diego, CA) was used to determine if

the difference between treated and control is significant.

3. Results

3.1. Linearity, recovery and detection limits

Calibration standard curves for 2'-deoxyguanosine and 8-OHdG was obtained for a concentration ranged between 1–10 ng (Fig. 2). Recovery of 2-deoxyguanosine and 8-hydroxy-2-deoxyguanosine from urine samples was determined for concentrations ranged between 1–100 ng. Average percentage recoveries were $84.8 \pm 9.2\%$ to $75.5 \pm 6.8\%$ for 2'-deoxyguanosine and 8-hydroxy-2'-deoxyguanosine (8-OHdG), respectively. Limits of detection of 2-deoxyguanosine and 8-hydroxy-2-deoxyguanosine were 0.5 ng.

3.2. HPLC analysis

Fig. 3 and Fig. 4 show the chromatograms of standard and urine samples of 2'-deoxyguanosine and 8-hydroxy-2'-deoxyguanosine under described HPLC conditions. Retention times were 10.7 min, and 12.6 min for 2'-deoxyguanosine and 8-hydroxy-2'-deoxyguanosine, respectively.

3.3. Levels of 2-deoxyguanosine and 8-hydroxy-2-deoxyguanosine

A single dermal dose of 400 mg/kg of DEET caused significant increase in the urinary excretion of 8-hydroxy-2'-deoxyguanosine when applied, alone or in combination with a dermal dose of 1.3 mg/kg of permethrin. Amount of 8-hydroxy-2'-deoxyguanosine and 2'-deoxyguanosine was corrected according to total volume of the urine and body weight of the animal. A single dermal dose of 1.3 mg/kg of permethrin produced an increase in the levels of 8-hydroxy-2'-deoxyguanosine in rat urine that was not statistically significant (Fig. 5 and Fig. 6). Level of 8-hydroxy-2'-deoxyguanosine and 2'-deoxyguanosine was calculated based on the percentage recovery of each chemical.

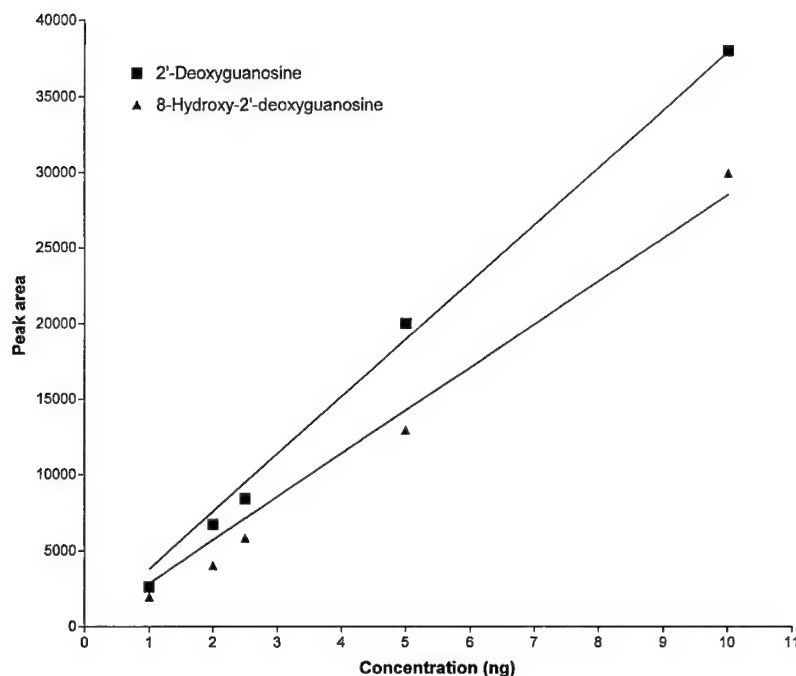


Fig. 2. Standard calibration curves of 2'-deoxyguanosine and 8-hydroxy-2'-deoxyguanosine.

3.4. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine if application of DEET and permethrin, alone or in combinations has caused significant increase in the levels of 8-OHdG excreted in the urine. DEET caused significant induction of 8-OHdG urinary levels ($P < 0.05$), while there was no significant difference of permethrin treatment and ethanol treated rats, or between DEET alone and DEET + permethrin treatment ($P < 0.1$). Prism program for Windows (GraphPad Software, Inc., San Diego, CA) was used for the analysis.

4. Discussion

Our results indicate that dermal administration of DEET to rats induced oxidative DNA damage as shown by increased urinary excretion of 8-hydroxy-2'-deoxyguanosine (8-OHdG) following DEET application. Both dermal doses of DEET when applied alone or in combination with per-

methrin caused similar induction. It seems that DEET caused free radical generation following application in rats, thus increasing the urinary levels of 8-OHdG compared to control urine samples. In this study we used a real-life exposure levels as determined by US Department of Defense (Personal Communications).

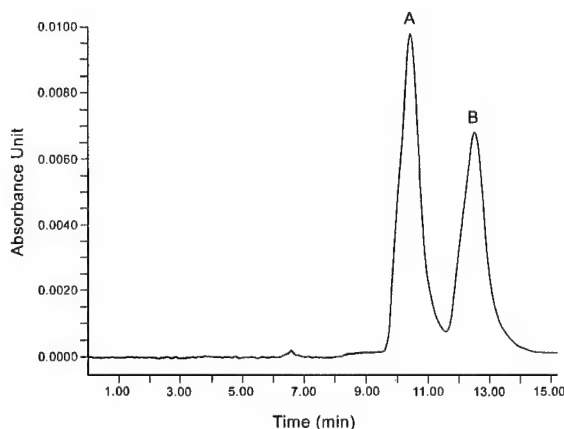


Fig. 3. HPLC chromatogram of standard (A) 2'-deoxyguanosine, (B) 8-hydroxy-2'-deoxyguanosine.

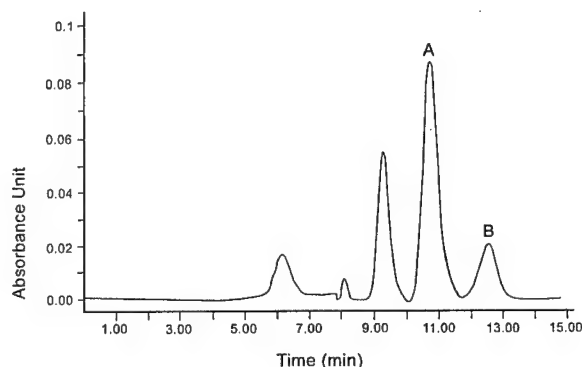


Fig. 4. HPLC chromatogram of urine sample of rats treated with a single dermal dose of 400 mg/kg of DEET (A) 2'-deoxyguanosine, (B) 8-hydroxy-2'-deoxyguanosine.

Although HPLC with UV detection is not the most sensitive method, separation of 8-OHdG from 2'-deoxyguanosine was achieved under the described HPLC condition. Our limit of detection was 0.5 ng compared to the more sensitive HPLC-MS, with detection of 8-OHdG in human urine of 0.2 ng/ml (Renner et al., 2000). Our ability to

detect both chemicals in most samples analyzed (2 ml urine) proved that our method is adequate in assessing DNA oxidative damage by measuring levels of 8-OHdG as a biomarker. The induction of urinary excretion of 8-OHdG in our experiment is consistent with previous studies following exposure to chemicals. Wellejus et al. (2000) reported increased of 8-OHdG excretion rate from 129 to 147 pmole 24 h after iv dose of iron in rats, and the urinary excretion 8-OHdG was significantly higher in rats received a single i.p. dose of 100 mg/kg of 2-nitropropane (Toraason et al., 1999). Also Yusof et al., (1999) reported that δ -aminolevulinic acid caused linear increase in 8-OHdG levels in Chinese hamster ovary, while incubation of *o*-phenylphenol metabolites, *o*-phenylhydroquinone and *o*-phenylbenzoquinone with Chinese hamster cells significantly enhanced of 8-OHdG concentration in nuclear DNA (Henschke et al., 2000). Similar finding was also reported following administration of 4 mg copper/kg rat that caused significant increased of 8-OHdG levels of DNA liver and kidney

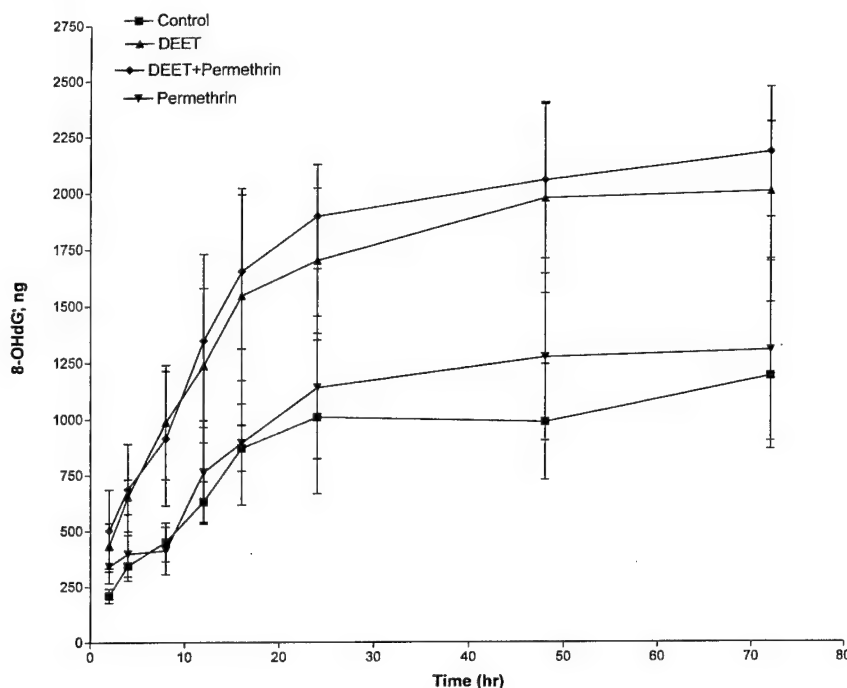


Fig. 5. Levels of 8-hydroxy-2'-deoxyguanosine in urine samples following a single dermal dose of DEET, and permethrin, alone and in combination.

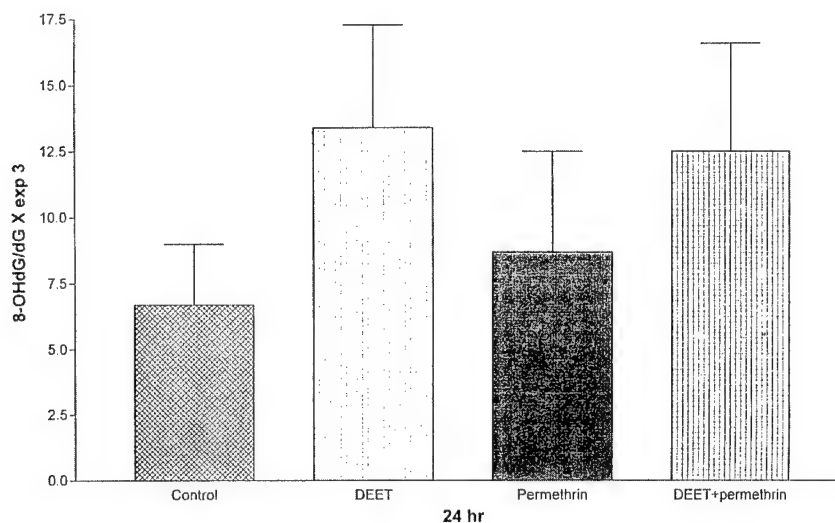


Fig. 6. Excretion of 2'-deoxyguanosine and 8-hydroxy-2'-deoxyguanosine in urine samples collected 24 h after a single dermal dose of DEET and permethrin, alone and in combination.

(Toyokuni and Sagripanti, 1994). Addition of dimethylformamide enhanced about 3–4 fold 8-OHdG formation induced by H_2O_2 and copper in calf thymus DNA (Midorikawa et al., 2000). Similar results were obtained in other species; exposure of fish to the insecticide dieldrin increased 8-OHdG levels of DNA liver (Rodriguez-Ariza et al., 1999), an increased in the amount of 8-OHdG was reported in diseases causing DNA oxidative damage. Jovanovic et al., (1998) detected about two fold increase in urinary excretion of 8-OHdG in 166 individuals with Down Syndrome compared to healthy subjects, and its levels were significantly higher in human sperm of infertile patients compared to healthy subjects (Shen et al., 1999). Other factors also increased 8-OHdG levels, such as in human leukocytes of smokers where the amount of 8-OHdG was significantly higher compared to non-smokers (Lodovici et al., 2000).

Urinary excretion of 8-OHdG reached a peak level after 24 h of dermal dose of DEET alone, or in combination with permethrin, then gradually leveled off. Peak levels of 8-OHdG in the excreted urine following treatment with DEET

were 1897 ng/24h. Loft et al., (1993) reported a range between 200–300 pmol/kg/24h of 8-OHdG in the urine of 169 humans where smokers excreted 50% higher than non-smoker subjects. Also Loft et al., (1998b) detected a levels between 400–2000 pmol/kg rat, that depended on type of diet.

Our results show that exposure of rats to a dermal dose of 1.3 mg/kg of permethrin did not cause significant increase of urinary excretion of 8-OHdG, suggesting that oxidative DNA damage was similar to that of control levels. The small dermal dose of 1.3 mg/kg of permethrin used and its slow dermal absorption compared to DEET might have resulted in a small concentration below the threshold level to reach the DNA target. No published data reported that pyrethroides significantly increased free radical formation. Umemura et al. (2000) reported no increased concentration of 8-OHdG levels in the kidney nuclear DNA following sub-chronic exposure of rats to *p*-dichlorobenzene. In summary, dermal application of DEET, alone or in combination with dermal dose of permethrin increased levels of 8-OHdG in rats urine.

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Locomotor and Sensorimotor Performance Deficit in Rats following Exposure to Pyridostigmine Bromide, DEET, and Permethrin, Alone and in Combination

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Since their return from Persian Gulf War (PGW), many veterans have complained of symptoms including muscle and joint pain, ataxia, chronic fatigue, headache, and difficulty with concentration. The causes of the symptoms remain unknown. Because these veterans were exposed to a combination of chemicals including pyridostigmine bromide (PB), DEET, and permethrin, we investigated the effects of these agents, alone and in combination, on the sensorimotor behavior and central cholinergic system of rats. Male Sprague-Dawley rats (200–250 gm) were treated with DEET (40 mg/kg, dermal) or permethrin (0.13 mg/kg, dermal), alone and in combination with PB (1.3 mg/kg, oral, last 15 days only), for 45 days. Sensorimotor ability was assessed by a battery of behavioral tests that included beam-walk score, beam-walk time, incline plane performance, and forepaw grip on days 30 and 45 following the treatment. On day 45 the animals were sacrificed, and plasma and CNS cholinesterase, and brain choline acetyl transferase, muscarinic and nicotinic acetylcholine receptors were evaluated. Animals treated with PB, alone or in combination with DEET and permethrin, showed a significant deficit in beam-walk score as well as beam-walk time as compared with controls. Treatment with either DEET or permethrin, alone or in combination with each other, did not have a significant effect on beam-walk score. All chemicals, alone or in combination, resulted in a significant impairment in incline plane testing on days 30 and 45 following treatment. Treatment with PB, DEET, or permethrin alone did not have any inhibitory effect on plasma or brain cholinesterase activities, except that PB alone caused moderate inhibition in midbrain acetylcholinesterase (AChE) activity. Treatment with permethrin alone caused significant increase in cortical and cerebellar AChE activity. A combination of DEET and permethrin or PB and DEET led to significant decrease in AChE activity in brainstem and midbrain and brainstem, respectively. A significant decrease in brainstem AChE activity was observed following combined exposure to PB and permethrin. Coexposure with PB, DEET, and permethrin resulted in significant inhibition in AChE in brainstem and midbrain. No effect was observed on

choline acetyl transferase activity in brainstem or cortex, except combined exposure to PB, DEET, and permethrin caused a slight but significant increase in cortical choline acetyltransferase activity. Treatment with PB, DEET, and permethrin alone caused a significant increase in ligand binding for m2 muscarinic acetylcholine receptor (mAChR) in the cortex. Coexposure to PB, DEET, and permethrin did not have any effect over that of PB-induced increase in ligand binding. There was no significant change in ligand binding for nicotinic acetylcholine receptor (nAChR) associated with treatment with the chemical alone; a combination of PB and DEET or coexposure with PB, DEET, and permethrin caused a significant increase in nAChR ligand binding in the cortex. Thus, these results suggest that exposure to physiologically relevant doses of PB, DEET, and permethrin, alone or in combination, leads to neurobehavioral deficits and region-specific alterations in AChE and acetylcholine receptors.

Key Words: Persian Gulf War; sensorimotor; pyridostigmine bromide; DEET; permethrin; combined exposure; CNS.

Since their return from the war, many Persian Gulf War (PGW) veterans have complained of symptoms including chronic fatigue, muscle and joint pain, ataxia, rash, headache, difficulty concentrating, forgetfulness, and irritability (Institute of Medicine, 1995). Haley *et al.* (1997a,b), used epidemiological analyses to characterize these symptoms into six syndromes. The veterans in the PGW were exposed to a unique combination of biological, chemical, and psychological environments. Combinations of chemical exposures included a variety of pesticides such as DEET and permethrin (Institute of Medicine, 1995). Additionally, these veterans were given a course of twenty-one 30-mg tablets of pyridostigmine bromide (PB) as prophylactic treatment to protect against organophosphate (OP) nerve agents (Persian Gulf Veterans Coordinating Board, 1995). PB is viewed to be relatively safe at the given dose.

PB is a quaternary dimethyl carbamate used as a treatment for myasthenia gravis at a higher dose range than what was

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given to PGW veterans (Breyer-Pfaff *et al.*, 1985, 1990). PB reversibly inhibits 30–40% of the AChE in the peripheral nervous system, thus limiting irreversible inhibition of the enzyme by nerve agents (Blick *et al.*, 1991). AChE activity is restored following spontaneous decarbamylation resulting in near-normal neuromuscular and autonomic functions (Blick *et al.*, 1991). Toxic symptoms associated with PB overdose results from overstimulation of nicotinic and muscarinic receptors in the peripheral nervous system, resulting in exaggerated cholinergic effects such as muscle fasciculations, cramps, weakness, muscle twitching, tremor, respiratory difficulty, gastrointestinal tract disturbances, and paralysis (Abou-Donia *et al.*, 1996; McCain *et al.*, 1997). With severe intoxication, death may occur because of asphyxia. Central nervous system effects of PB are not expected unless BBB permeability is compromised. The positive charge on the quaternary pyridinium nitrogen prevents PB from crossing the intact BBB (Birtley *et al.*, 1966).

The insect repellent *N,N*-diethyl-*m*-toluamide (DEET) and the insecticide pyrethroid permethrin 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl) methyl ester have been used extensively by humans since their introduction. DEET is commonly used as a repellent against mosquitoes, flies, ticks, and other insects (McConnel *et al.*, 1986; Robbins and Cherniack, 1986). However, extensive and repeated topical DEET applications can cause human poisoning, including death (Edwards and Johnson, 1987; Gryboski *et al.*, 1961; Roland *et al.*, 1985). The symptoms associated with DEET poisoning include tremors, restlessness, difficulty with speech, seizures, impairment of cognitive function, and coma (McConnel *et al.*, 1986). DEET efficiently crosses the dermal barrier and may localize to dermal fat deposits (Blomquist and Thorsell, 1977; Snodgrass *et al.*, 1982). Although the exact mechanisms of DEET toxicity are not known, extremely high levels of DEET exposure cause demyelination and spongiform myelinopathy in the rat (Verschoyle *et al.*, 1992).

Permethrin is a type I synthetic pyrethroid insecticide that exists in four different stereoisomers (Casida *et al.*, 1983). It provides insecticidal activity for several weeks following a single application and is used in a variety of public buildings, industrial premises, and private dwellings to control fleas, flies, mites, and cockroaches. Permethrin intoxication results as a consequence of modification of sodium channels, leading to prolonged depolarization and repetitive discharges in presynaptic nerve fibers after a single stimulus (Narahashi, 1985). This repetitive nerve action is associated with tremor, hyperactivity, ataxia, convulsions, and in some cases to paralysis. Permethrin is detoxified by ester hydrolysis in the blood and most tissues.

We previously reported that concurrent exposure to relatively large doses of PB, DEET, and permethrin in hens resulted in neurotoxic effects greater than those produced by exposure to the single components (Abou-Donia *et al.*, 1996). In the present study we have extended these observations to

include doses similar to levels of human exposure. We evaluated whether PB would enhance the neurotoxic effects caused by low-dose, combined exposure to DEET and permethrin. These results suggest that treatment with PB, DEET, and permethrin, alone or in combination, causes a significant impairment in sensorimotor abilities and region-specific effects on brain AChE and mAChR.

MATERIALS AND METHODS

Chemicals. Technical-grade ($\geq 93.6\%$) permethrin 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl) methyl ester was obtained from Roussel Uclaf Corp., Pasadena, TX. DEET (99.7% *N,N*-diethyl-*m*-toluamide), pyridostigmine bromide ($\geq 99\%$, 3-dimethylamino carbonyloxy-*N*-methylpyridinium bromide), acetylthiocholine iodide, butyrylthiocholine iodide, atropine, and nicotine were purchased from Sigma Chemical Co., St. Louis, MO. 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) was purchased from Aldrich. The inhibitor, 1,5-bis-(*N*-allyl-*N*,*N*-dimethyl-4-ammonium phenyl) pentan-3-one dibromide (BW284C51) was obtained from Sigma Chemical Co., St. Louis MO. [^3H]AF-DX 384, sp. activity 106 $\mu\text{Ci}/\text{mmol}$, [^3H]cytosine, sp. activity 32 nCi/pmol, and [^3H]acetyl CoA, sp. activity 12 $\mu\text{Ci}/\text{mmol}$ were purchased from New England Nuclear, Boston, MA. All other chemicals and reagents were of highest purity available from commercial sources.

Animals. Male Sprague-Dawley rats weighing 225–250 g were obtained from Zivic-Miller Laboratories, Allison Park, PA, and housed at Duke University Medical Center vivarium. The animals were randomly assigned to control and treatment groups and housed at 21–23°C with a 12-h light/dark cycle. They were supplied with food and water *ad libitum*. The rats were allowed to adjust to their environment for a week before starting the treatment. Animal care was in accordance with institutional guidelines.

Treatment. The dosages of chemicals used were as follows: PB (1.3 mg/kg/day in water, oral), DEET (40 mg/kg/day in 70% ethanol, dermal), and permethrin (0.13 mg/kg/day in 70% ethanol, dermal). These doses of PB, DEET, and permethrin correspond to real-life exposure to military personnel during the PGW. Oral doses were given by gavage, while dermal applications were applied to the back of the neck on a 1-in² area preshaved with electric clippers. Groups with five animals each were treated as described below. Animals were sacrificed 24 h after treatment with the last dose.

- Control: animals receiving daily dermal dose of 70% ethanol and water for the last 15 days of the experiment
- Pyridostigmine bromide for the last 15 days of the experiment
- DEET daily for 45 days
- Permethrin daily for 45 days
- DEET and permethrin daily for 45 days
- DEET for 30 days, then PB + DEET for the last 15 days
- Permethrin for 30 days, then PB + permethrin for the last 15 days
- DEET and permethrin for 30 days, then PB + DEET + permethrin for the last 15 days

Behavioral Studies. A battery of standardized tests was employed on days 30 and 45 following the treatment. These behavioral tests were designed to measure sensorimotor reflexes, motor strength, and coordinated gait (Bederson *et al.*, 1986; Goldstein, 1993; Markgraf *et al.*, 1992). All behavioral testing was performed by a trained observer blind to the treatment status of the animal and was carried out in a soundproof room with subdued lighting (less than 10.76 lumens/m², ambient light). Rats were handled for 2 min daily for 5 days during the week prior to behavioral testing.

Postural Reflexes

Description. Rats were held gently by the tail, one meter above the floor, and observed for forelimb extension. Normal rats extend both forelimbs.

Consistent flexion of the forelimb is an abnormal response. Rats with consistent forelimb flexion are then further assessed by being placed on a large sheet of plastic-coated paper that can be gripped with the forepaws. With the tail held, gentle lateral pressure was applied behind the shoulder of the rat until the forelimb slid several inches. The maneuver was repeated five times in each direction. Normal rats resist lateral pressure by gripping the coated paper.

Scoring. Grade 0: rats without evidence of consistent forelimb flexion when held above the floor; grade 1: rats with consistent forelimb flexion; grade 2: otherwise grade 1 rats that do not resist lateral pressure on at least three of five trials in either direction.

Limb Placing

Description. Visual, tactile, and proprioceptive forelimb placing responses were examined. For visual placing, rats were held in the hands of the examiner 10 cm above the tabletop, with forelimbs hanging free. The rats were then slowly tilted toward the table. Intact rats reach toward the table with both forepaws. For tactile placing, the dorsal and then lateral portions of the forepaws were touched to the table edge. Intact rats immediately place the paw on the surface of the table. Proprioceptive placing was tested by pushing the forepaw onto the table edge. Care was taken to avoid the vibrissae touching the table.

Scoring. For each test: grade 0, the placing response is immediate; grade 1, the placing response is slow or delayed; grade 2, the placing response does not occur within 2 s.

Orienting to Vibrissae Touch

Description. The rat was placed atop an inverted polycarbonate cage and allowed 1 min for habituation. Its vibrissae were then touched with a cotton-tipped swab.

Scoring. Grade 0: rat orients to the side of the probe on at least two of three trials from each side; grade 1, rat fails to orient on at least two of three trials on either side.

Grip Time. Forepaw grip time of the rats was assessed by having them hang from a 5 mm diameter wood dowel gripped with both forepaws. Time to release their grip was recorded in seconds.

Beam-Walking and Beam Score

Description. The testing apparatus was a 2.5 × 122 cm wooden beam elevated 75.5 cm above the floor with wooden supports. A 20 × 25 × 24 cm goal box with a 9.5 cm opening is located at one end of the beam. A switch-activated source of bright light (75 watt Tungsten bulb) and white noise (41 dB at 8000 Hz, 58 dB at 4000 Hz, 56 dB at 2000 Hz, 56 dB at 1000 Hz, 58 dB at 500 Hz, and 52 dB at 250 Hz SPL at the center of the frequency at each octave band) were located at the start end of the beam and served as avoidance stimuli. The rats were first trained with a series of three approximate trials. Rats are readily trained to perform the beam-walking task (Goldstein, 1993).

Scoring. Both the latency until the animal's nose entered the goal box (up to 90 s) and the use of the hind paw to aid locomotion were recorded. Beam-walking ability was measured with a seven-point scoring system scale as previously described (Goldstein, 1993): 1, the rat is unable to place the affected hindpaw on the horizontal surface of the beam; 2, the rat places the hindpaw on the horizontal surface of the beam and maintains balance for at least 5 s; 3, the rat traverses the beam while dragging the affected hindpaw; 4, the rat traverses the beam and at least once places the affected hindpaw on the horizontal surface of the beam; 5, the rat crosses the beam and places the affected hindlimb on the horizontal surface of the beam to aid less than half its steps; 6, the rat uses the affected hindpaw to aid more than half its steps; and 7, the rat traverses the beam with no more than two footslips. In addition, the latency until the animal's nose enters the goal box (up to 90 s) is recorded for the final trial. Rats that fell off the beam were assigned latencies of 90 s.

Incline Plane

Description. The rats were placed on a flat plane in the horizontal position, with the head facing the side of the board to be raised according to the method described by Yonemori *et al.* (1998). The board was slowly rotated to the vertical position. Two trials were performed for each testing session.

Scoring. The angle that the rat began to slip downward was recorded. The results of two trials were averaged at each time point.

Statistical Analysis. For continuous data (beam-walk time, beam-walk score, grip time, and incline plane), groups were compared by two-way ANOVA, with repeated measures as appropriate. The significance of post hoc pairwise comparison was determined with Fisher's LSD tests. For nonparametric data (postural reflexes, limb placing, and vibrissal touch), comparisons across treatment groups were made with the Kruskal-Wallis test. If the Kruskal-Wallis test indicated a significant difference among the groups, Dunn's procedure would be applied to the ranks of the data to determine the significance of post hoc, pairwise comparisons.

Assays

Acetylcholinesterase and butyrylcholinesterase assays. Brain acetylcholinesterase (AChE) and plasma cholinesterase (BChE) activities were measured by the Ellman assay (Ellman *et al.*, 1961). For AChE assays, dissected brain regions were homogenized in Ellman buffer and centrifuged for 5 min at 5000 × *g*; the resulting supernatant was used for AChE analysis. AChE activity was measured using acetylthiocholine as substrate in a Molecular Devices UV Max Kinetic Microplate Reader at 412 nm. 5,5'-Dithio-bis-2-nitrobenzoic acid (DTNB) was used as the color reagent as described by Abou-Donia *et al.* (1996). Protein concentrations in tissue samples and plasma were determined by the method of Smith *et al.* (1985).

Choline acetyl transferase. Choline acetyl transferase activity in brain was determined using methods by Fonnum (1975).

Muscarinic acetylcholine receptor binding. For the assay of the ligand binding for m2 mAChR, the tissue was homogenized in 10 mM phosphate buffer, pH 7.4, and centrifuged at 40,000 × *g* for 10 min. The membranes were suspended in the same buffer at a protein concentration of 1.25–2.5 mg/ml as described by Huff *et al.* (1994), and the ligand binding was carried out according to Slotkin *et al.* (1999). The m2 mAChR binding was carried out by using m2 mAChR-specific ligand, [³H]JAF-DX 384 at room temperature for 60 min. Results are presented as specific receptor binding (dpm)/mg protein (percent of control).

Nicotinic acetylcholine receptor binding. [³H]Cytisine was used as specific ligand for nAChR according to the method described by Slotkin *et al.* (1999). An aliquot of membrane preparation containing ~200 µg protein was used to carry out the incubation with 1 nM [³H]cytisine at 4°C for 75 min. Results are presented as specific receptor binding (dpm)/mg protein (percent of control).

Statistics. For biochemical assays, treatment groups were compared to control groups by two-way unpaired *t*-test using Prism GraphPad™ software, and results were plotted using Excel graphics for Macintosh.

RESULTS

General Health and Clinical Condition

There were no overt clinical signs of toxicity observed throughout the study except for occasional diarrhea in rats receiving DEET. There were no significant differences in weights between the treatment groups throughout the study.

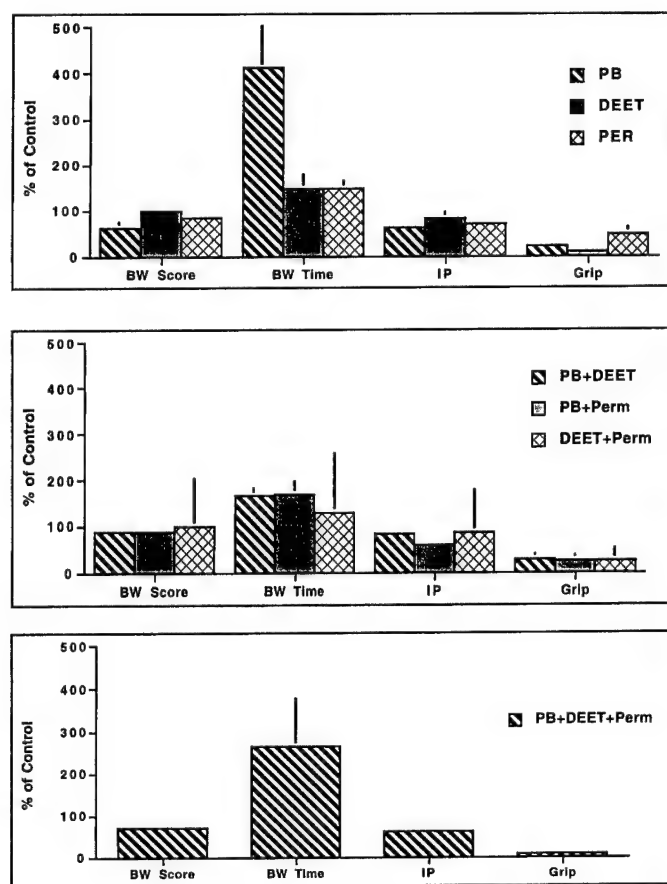


FIG. 1. Effect of treatment with PB, DEET, and permethrin, alone or in combination, on sensorimotor performance on day 30 of the beginning of treatment with DEET or permethrin. The animals were treated with PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal), and permethrin (0.13 mg/kg, dermal) as described in Materials and Methods. The animals were examined blindfolded for beam-walk score (BW score), beam-walk time (BW time), incline plane (IP), and grip response. The data were computed and detailed statistical evaluations were carried out as described in the Results section. Top panel: data obtained from exposure with single chemical. Middle panel: data obtained from exposure with two chemicals. Bottom panel: data obtained from exposure with three chemicals. For comparison purposes, the data are presented as means \pm SE (percent of control).

Effect of DEET, Permethrin or PB, Alone or in Combination, on Sensorimotor Function

A battery of behavioral tests was carried out to assess the sensorimotor function. We focused on sensorimotor ability because the majority of PGW veterans' complaints related to muscle and joint pain, fatigue, disorientation, and ataxia. Animals were tested on days 30 and 45 from the beginning of the treatment. Although statistical analyses were performed on the actual data, for the sake of comparability, the data obtained from beam-walk score, beam-walk time, incline plane, and grip time are presented as a percent of control. In the figures, error bars reflect SEM based on the raw data and recalculated to reflect percent of control. Figure 1 represents the measure-

ments carried out on day 30, and Figure 2 represents the activity measured on day 45 following the beginning of the treatment with DEET and permethrin.

There was no effect of any of the drugs, alone or in combination, on postural reflexes, limb placing, or vibrissae touch (data not shown). Control animals consistently showed completely normal performance (Kruskal-Wallis, $p > 0.05$ for each comparison). For beam-walk score, two-way repeated measures ANOVA showed a significant effect of treatment group (ANOVA $F_{7,70} = 8.4$, $p < 0.0001$) and a significant treatment group \times time interaction effect (ANOVA $F_{1,7} = 2.3$, $p = 0.03$). Given alone, PB, but not DEET or permethrin, differed significantly from control (Fisher LSD, $p = 0.0001$). The poorest performance was seen in rats that received all three drugs and

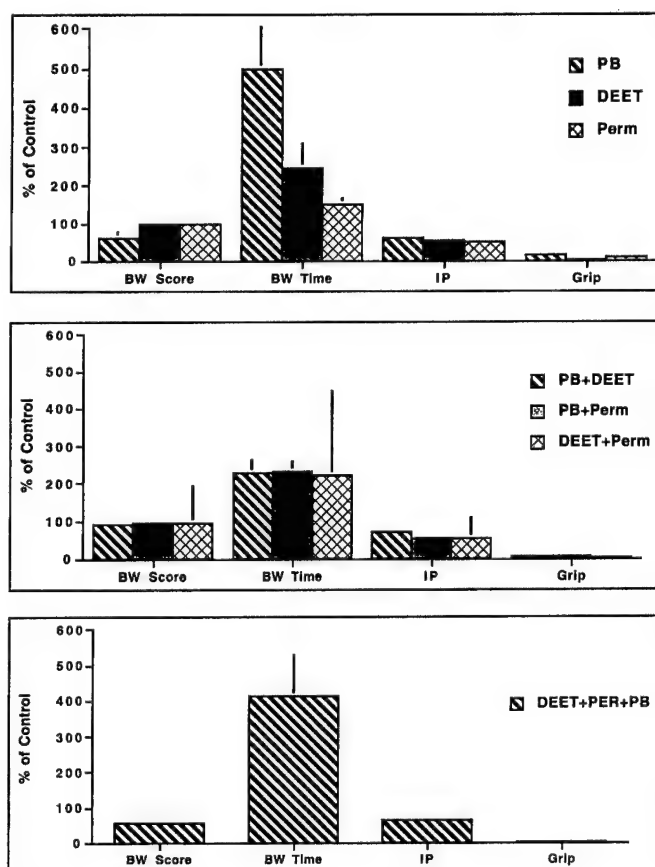


FIG. 2. Effect of treatment with PB, DEET, and permethrin, alone or in combination, on sensorimotor and locomotor performance on day 45 of the beginning of treatment with DEET or permethrin. The animals were treated with PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal), and permethrin (0.13 mg/kg, dermal) as described in Materials and Methods. The animals were examined blindfolded for beam-walk score (BW score), beam-walk time (BW time), incline plane (IP), and grip response. The data were computed and detailed statistical evaluations were carried out as described in the Results section. Top panel: data obtained from exposure with single chemical. Middle panel: data obtained from exposure with two chemicals. Bottom panel: data obtained from exposure with three chemicals. For comparison purposes, the data are presented as means \pm SE (percent of control).

those receiving PB. There was no significant difference in performance between rats given PB and those given DEET, permethrin, and PB (Fisher LSD, $p = 0.71$).

For beam-walking time, two-way repeated measures ANOVA showed a significant effect of treatment group (ANOVA $F_{7,70} = 8.4$, $p = 0.006$) but no treatment group \times time interaction (ANOVA $F_{1,7} = 0.7$, $p = 0.65$). Given alone, pyridostigmine, but not DEET or permethrin, differed significantly from control (Fisher LSD, $p = 0.0002$). There was not a significant difference between rats given PB and those given DEET, permethrin, and PB (Fisher LSD, $p = 0.20$).

For incline plane performance (Figs. 1 and 2), two-way repeated measures ANOVA showed a significant effect of treatment group (ANOVA $F_{7,70} = 38.1$, $p < 0.0001$) and a significant treatment group \times time interaction (ANOVA $F_{1,7} = 4.5$, $p = 0.004$). All drugs given alone differed significantly from control (Fisher LSD, $p < 0.0001$). There was no significant difference between rats given PB and those given DEET, permethrin, and PB (Fisher LSD, $p > 0.9$).

Finally, for forepaw grip time (Figs. 1 and 2), two-way repeated measures ANOVA showed a significant effect of treatment group (ANOVA $F_{7,70} = 44.5$, $p = 0.001$) and a significant treatment group \times time interaction (ANOVA $F_{1,7} = 4.9$, $p = 0.001$). All drugs given alone differed significantly from control (Fisher LSD, $p < 0.0001$). There was no significant difference between rats given PB and those given DEET, permethrin, and PB (Fisher LSD, $p = 0.06$).

A similar decreasing but insignificant trend in horizontal and vertical movement was observed in the animals treated with the combination of PB, DEET, and permethrin (data not shown).

In summary, each drug given alone had a significant behavioral effect, which tended to become more evident over time. There also was no significant difference on any parameter between rats given PB alone and those given DEET, permethrin, and PB. Most significant deficits were observed in animals given PB or a combination of PB with other chemicals.

Effect of PB, DEET, and Permethrin, Alone or in Combination, on Plasma and Brain Cholinesterase Activities

Plasma BChE and AChE activities in cortex, brainstem, midbrain, and cerebellum from the animals treated with PB, DEET, and permethrin, alone or in combination, were assayed. Data on the effects of single-chemical treatment are presented in Figure 3 (top panel). Treatment with PB, alone or in combination with DEET and permethrin, caused slight but insignificant inhibition ($\sim 96\%$ of controls) of plasma BChE activity. Treatment with DEET, alone or in combination with PB or permethrin, caused a variable but insignificant increase ($\sim 125\text{--}140\%$ of control) in BChE activity. Treatment with permethrin, either alone or in combination, did not have any effect on BChE activity.

Treatment with PB alone inhibited the AChE activity in midbrain ($\sim 60\%$ of control, $p < 0.04$) and produced no

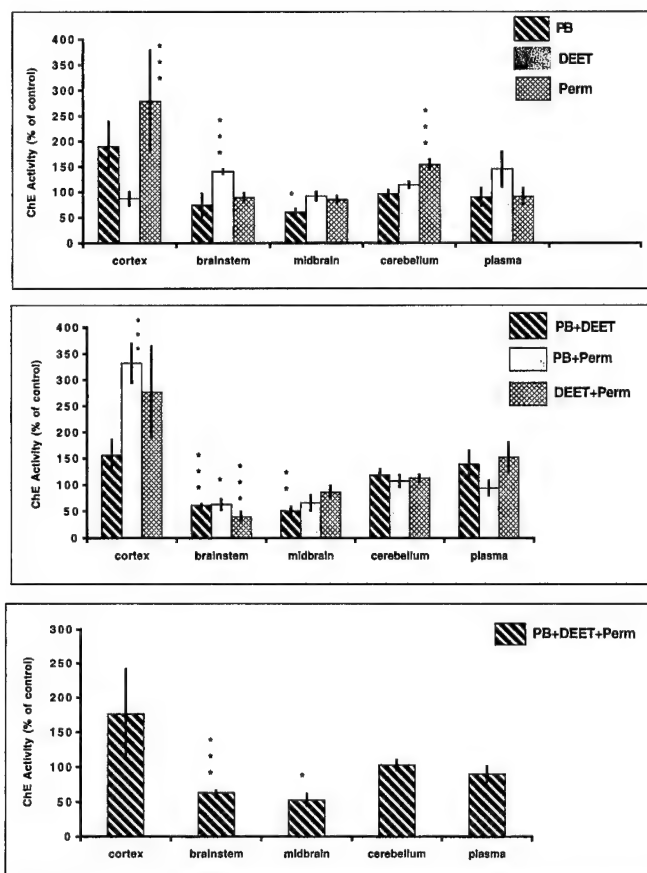


FIG. 3. Effect of treatment with PB, DEET, and permethrin, alone or in combination, on brain regional AChE and plasma BChE activities. The animals were treated with PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal), and permethrin (0.13 mg/kg, dermal) as described in Materials and Methods. Treatment with PB was given on the last 15 days of the experiment. The details of the treatment and determination of enzyme activity are elaborated in Materials and Methods. Top panel: data obtained from exposure with single chemical. Middle panel: data obtained from exposure with two chemicals. Bottom panel: data obtained from exposure with three chemicals. Data are presented as means \pm SE (percent of control). p value: * < 0.04 , ** < 0.01 , *** < 0.001 .

significant changes in enzyme activity in brainstem, cortex, and cerebellum. DEET or permethrin treatment alone did not cause any significant inhibitory effect on brain region AChE activities. Instead, DEET treatment alone caused a significant increase ($\sim 140\%$ of control) in brainstem enzyme activity, and permethrin alone caused a significant increase ($\sim 278\%$ of control) in cortical enzyme activity ($p < 0.001$).

Data on combination of two chemicals are presented in Figure 3 (middle panel). A combination of PB and DEET treatment resulted in significant AChE inhibition in the brainstem and midbrain ($p < 0.001$ and 0.01 , respectively). Combined exposure with PB and permethrin resulted in significant inhibition ($\sim 67\%$ of control, $p < 0.04$) in brainstem AChE activity, whereas cortex activity remained significantly increased. Combined exposure with DEET and permethrin re-

sulted in significant inhibition ($p < 0.001$) in brainstem AChE activity, whereas other regions did not show any change in the activity that was different than individual chemical alone. This was consistent with our previous studies (Abou-Donia *et al.*, 1996).

Data presented in Figure 3 (bottom panel) indicate that brainstem and midbrain AChE activity was significantly inhibited when animals were exposed to the combination of PB, DEET, and permethrin (~ 60 – 65% of control, $p < 0.001$ and 0.04 , respectively). The magnitude of inhibition is similar to that observed when animals were exposed to a combination of PB and DEET, suggesting that PB under these treatment conditions has the potential to inhibit the brainstem and midbrain AChE activity.

Effect of PB, DEET, and Permethrin, Alone or in Combination, on Brain Choline Acetyl Transferase (ChAT) Activity

Choline acetyl transferase (ChAT) catalyzes the final step in the biosynthesis of acetylcholine by facilitating the irreversible transfer of acetyl groups of acetylCoA to choline. In view of the changes induced by PB in CNS on AChE activity, and because PB-induced inhibition of AChE is reversible and short-lived (Watts and Wilkinson, 1977), we argued that there may exist alternative mechanisms of acetylcholine buildup. Therefore, we studied the effects of treatment with PB, DEET, and permethrin, alone or in combination, on ChAT activity in brainstem and cortex. In the CNS, the majority of ChAT activity is localized in brainstem and cortex (Wu and Hersh, 1994). Data in Figure 4 represent the enzyme activity in the cortex and brainstem. Treatment with PB and permethrin alone did not have any significant effect on brainstem or cortex enzyme activities, whereas DEET treatment caused a significant increase in the enzyme activity in the cortex ($p < 0.001$). Combined exposure of PB and permethrin caused a significant increase ($p < 0.001$) in ChAT activity in the cortex. However, coexposure with PB, DEET, and permethrin did not result in any significant change in enzyme activity in either region.

Effect of PB, DEET, and Permethrin, Alone or in Combination, on m2 Muscarinic and Nicotinic Acetylcholine Receptor Activity

In order to evaluate the effect of treatment with PB, DEET, and permethrin, alone or in combination, on muscarinic receptor, ligand binding studies were carried out with membrane preparations using m2-specific ligand [3 H]AFDX in cortex, brainstem, midbrain, and cerebellum. The data presented in Figure 5 indicate that PB treatment alone caused a significant increase in ligand binding density in the cortex ($\sim 165\%$ of control, $p < 0.001$) and no effect in midbrain and brainstem. Treatment with DEET or permethrin alone caused a significant increase in ligand binding density in the cortex. A similar increase in ligand binding in the cortex was observed with combined exposure of PB and DEET, and DEET and per-

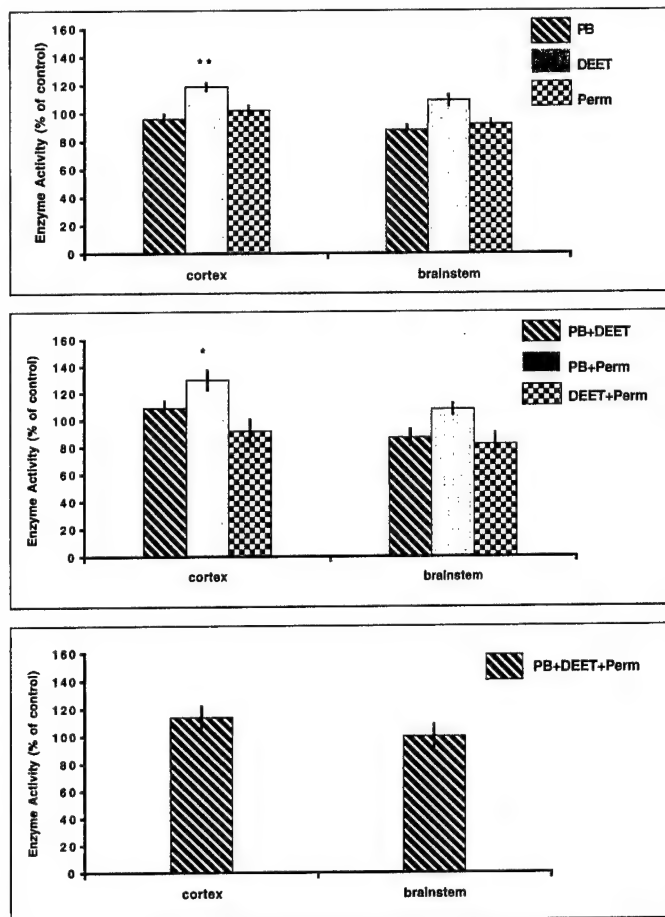


FIG. 4. Effect of treatment with PB, DEET, and permethrin, alone or in combination, on cortex and brainstem choline acetyl transferase activity. The animals were treated with PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal), and permethrin (0.13 mg/kg, dermal) as described in Materials and Methods. Treatment with PB was given on the last 15 days of the experiment. The details of the treatment and determination of enzyme activity are elaborated in Materials and Methods. Top panel: data obtained from exposure with single chemical. Middle panel: data obtained from exposure with two chemicals. Bottom panel: data obtained from exposure with three chemicals. Data are presented as means \pm SE (percent of control). p value: * < 0.01 , ** < 0.001 .

methrin. There was a significant increase ($\sim 150\%$, $p < 0.006$) in cerebellum in the animals treated with PB and permethrin. Combined exposure with PB, DEET, and permethrin led to significant increase in ligand binding only in cortex ($p < 0.001$).

Ligand binding for nicotinic acetylcholine receptors using [3 H]cytisine was carried out in the cortex membranes prepared from the animals treated with PB, DEET, and permethrin, alone or in combination. The data presented in Figure 6 show that treatment with PB, DEET, and permethrin alone did not cause any significant change in ligand binding. Treatment with DEET in combination with PB or permethrin led to a significant increase in the ligand binding density ($\sim 125\%$ of control, $p < 0.03$). Coexposure with PB, DEET, and permethrin caused a significant increase ($\sim 138\%$, $p < 0.03$) in ligand binding.

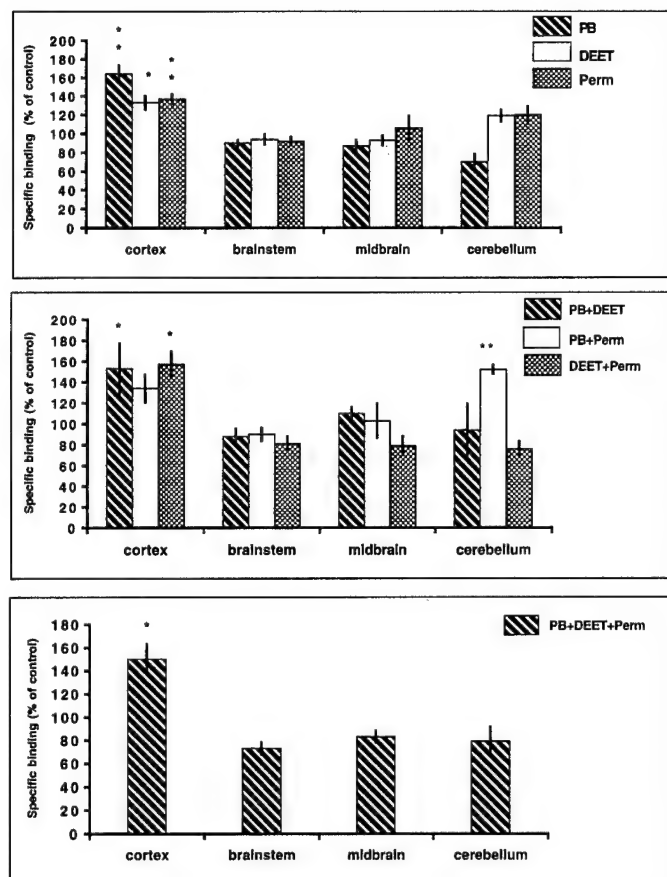


FIG. 5. Effect of treatment with PB, DEET, and permethrin, alone or in combination, on m2 muscarinic acetylcholine receptor ligand binding in brain regions. The animals were treated with PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal), and permethrin (0.13 mg/kg, dermal) as described in Materials and Methods. Treatment with PB was given on the last 15 days of the experiment. The details of the treatment, membrane preparation, and [3 H]AFDX384 binding assay are elaborated in Materials and Methods. Top panel: data obtained from exposure with single chemical. Middle panel: data obtained from exposure with two chemicals. Bottom panel: data obtained from exposure with three chemicals. Data are presented as means \pm SE (percent of control). p value: * < 0.01, ** < 0.006.

DISCUSSION

The present study examined effects of exposure to physiologically relevant doses of PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal), and permethrin (0.13 mg/kg, dermal), alone and in combination, on sensorimotor behavior and cholinergic system. The results suggest that exposure to these chemicals, alone or in combination, causes significant sensorimotor deficit. Furthermore, these data also suggest that treatment with PB, DEET, and permethrin, alone and in combination, causes differential regulation of AChE and m2 muscarinic and nicotinic acetylcholine receptors in the CNS.

The anatomical and molecular bases of the behavioral effects observed in the present study are complex. Different lesion studies have shown that severe sensorimotor impairment

occurs in the animals with lesions of anteromedial and caudal forelimb cortex (Barth *et al.*, 1990). Similarly, studies with bilateral large lesions in the rat somatic sensorimotor cortex have shown impairment in limb-placing response. Additionally, it has also been suggested that limb placing is a function of corticospinal tract (Hicks and D'Amato, 1975). Thus, it is possible that treatment with PB, DEET, and permethrin, alone or in combination, could affect these innervations as well as innervations in other brain regions, and as a consequence, sensorimotor deficit may occur after prolonged exposure.

Beam-walking performance is an integrated form of behavior necessitating pertinent levels of consciousness, memory, sensorimotor, and cortical functions mediated by cortical area, and it has been suggested that an injury to cortex is reflected by a deficit in beam-walk task. A role for norepinephrine (NE) has strongly been proposed in the deficit caused by cortical injury (Boyeson *et al.*, 1992; Goldstein, 1995). It has been suggested

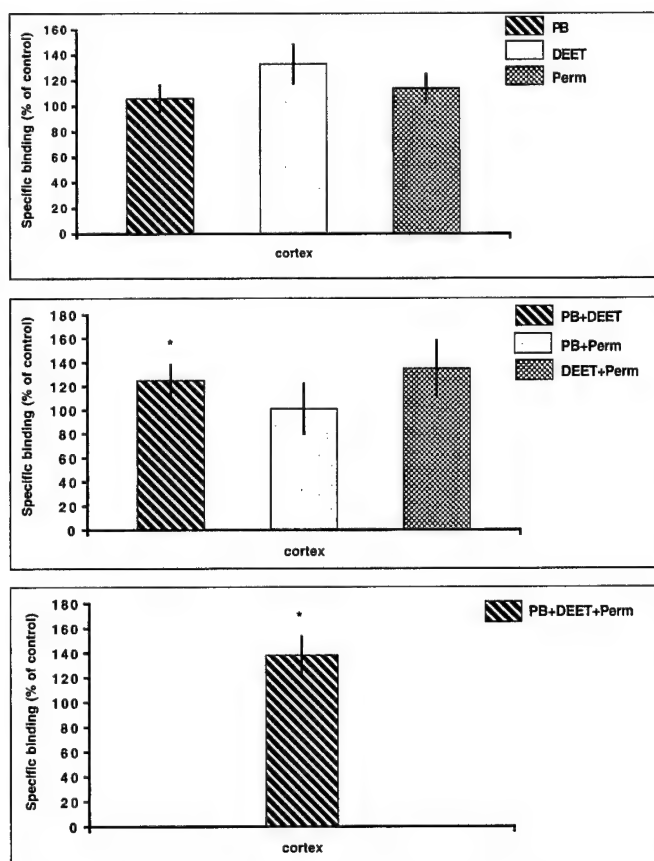


FIG. 6. Effect of treatment with PB, DEET, and permethrin, alone or in combination, on nicotinic acetylcholine receptor ligand binding in cortex. The animals were treated with PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal), and permethrin (0.13 mg/kg, dermal) as described in Materials and Methods. Treatment with PB was given on the last 15 days of the experiment. The details of the treatment, membrane preparation, and [3 H]cytisine binding assay are elaborated in Materials and Methods. Top panel: data obtained from exposure with single chemical. Middle panel: data obtained from exposure with two chemicals. Bottom panel: data obtained from exposure with three chemicals. Data are presented as means \pm SE (percent of control). p value: * < 0.03.

that NE facilitates the recovery from locomotor deficit by alleviating injury-induced decrease and turnover of NE levels in the cortex (Boyeson and Feeney, 1990; Kikuchi *et al.*, 2000). NE originates from the locus coeruleus and is widely distributed in the CNS, including cerebral cortex, hippocampus, cerebellum, and spinal cord. Therefore, it is possible that treatment with PB, DEET, and permethrin, alone and in combination, might regulate the noradrenergic and other catecholaminergic pathway in these animals. However, in view of the complexity of the behavioral outcome, it is also possible that these deficits are the result of exposure of these chemicals on multiple regions in the brain.

PB has been used to protect against organophosphate nerve agent poisoning. It provides protection by shielding the peripheral AChE by reversibly binding to it. Because PB is a positively charged quaternary ion, it does not cross the BBB under ordinary circumstances. Thus, the toxic effects of PB are thought to be mediated through peripheral ACh nicotinic and muscarinic receptors (Albuquerque *et al.*, 1997). Indeed, Chaney *et al.* (1999) found that PB-induced seizures in the mouse were mediated via peripheral nervous system (PNS) muscarinic and nicotinic receptors. However, other studies also suggest that PB toxicity is mediated through CNS ACh receptors as well as through the PNS (Servatius *et al.*, 1998). Our results indicate that low-dose PB treatment for 15 days inhibited midbrain AChE activity, whereas plasma BChE activity showed little inhibition. It is possible that PB entry into the CNS and the consequent inhibition of AChE in the CNS may enhance the toxic potential of neurotoxic agents. It is not certain yet how PB could affect the CNS. It is possible that continuous treatment PB, alone or in combination with DEET and permethrin, could affect the BBB permeability, thus allowing PB to enter the CNS. Other studies also demonstrate that treatment with chemicals that cause inhibition of AChE lead to m2 mAChR up-regulation (Majocha and Baldessarini, 1984; Witt-Enderby *et al.*, 1995).

Some PGW veterans were exposed to a combination of pesticides and insecticides such as DEET and permethrin. In addition, they were exposed to PB because they were allowed to ingest twenty-one 30-mg tablets of PB. DEET is highly permeable to the skin and has been studied (Baynes *et al.*, 1997; Selim *et al.*, 1995) for its metabolism and toxicity. Permethrin has been used to impregnate the clothing of military personnel as protection against pestiferous and vector insects (Taplin and Meinking, 1990). It is possible that combined exposure to these chemicals would result in differential effects than exposure to single chemicals. Indeed our biochemical data show this phenomenon. Our data on cholinesterase suggest that DEET and permethrin alone do not inhibit the AChE activity in the CNS or plasma, whereas a combination with PB resulted in significant inhibition in brainstem and midbrain activity. These data are consistent with our previous studies in chickens (Abou-Donia *et al.*, 1996). An intriguing finding in our study is that treatment with permethrin alone

caused a significant increase in cortical and cerebellar AChE activity, whereas DEET treatment alone caused a significant increase in brainstem AChE activity. The combination treatment led to a significant inhibition of AChE activity in brainstem, suggesting that brainstem may be the most susceptible to combined exposure. This inhibition may be mediated by PB, which might gain entry in the CNS following coexposure with DEET and permethrin. Treatment with DEET or permethrin caused an increase in AChE activity that may be due to an increase in AChE protein levels. Although not universally accepted, an increase in AChE protein may reflect an increased axonal repair and synaptic modeling, as has been shown recently (Bigbee *et al.*, 2000; Guizzetti *et al.*, 1996; Sternfeld *et al.*, 1998). Therefore, it is possible DEET and permethrin treatment alone may cause subtle changes that are reflected in increased synaptic modeling and repair. The behavioral data on single chemicals substantiate this notion. Coexposure with PB, DEET, and permethrin together caused a significant inhibition in brainstem and midbrain AChE activity, suggesting that treatment with three chemicals together could lead to added neurotoxic effects.

Cholinergic system in the CNS plays an important role in learning and memory (Lena and Changeux, 1998; Levey *et al.*, 1995). We studied the receptor ligand binding for m2AChR and nicotinic AChR in the cortex. Based on our data, it appears that increased receptor ligand binding density for both of the receptors in the cortex in response to treatment with PB, DEET, and permethrin, alone or in combination, may be a compensatory mechanism for a reduced ability of these receptors to bind their respective ligands. It is known that treatment with muscarinic antagonists induces receptor up-regulation (Ben-Barak and Dudai, 1980; Coccini *et al.*, 2000; Majocha and Baldessarini, 1984; Smiley *et al.*, 1998). Wang *et al.* (1996) reported the regulation of muscarinic receptor by repeated treatment with nicotine. The up-regulation of cortex m2AChR may be related to an increase in the AChE levels in the cortex of the animals treated with DEET or permethrin. Increased AChE activity in the cortex suggests that ACh levels are depleted. It is possible that subsequent receptor up-regulation is a response to reduced neurotransmitter levels. Increased ligand binding for m2 muscarinic receptor results in the inhibition of adenylate cyclase activity through a pertussis toxin-sensitive G-protein, resulting in an inhibitory postsynaptic response (Brann *et al.*, 1993; Wess, 1996). The inhibitory nature of m2 receptor may have regulatory response on (GABA)ergic system in the cortex. It is known that cholinergic input in certain brain regions tonically inhibits (GABA)ergic system and is inhibitory to vasomotor glutamergic neurons. Thus, an increase in m2AChR in response to treatments with PB, DEET, and permethrin, alone or in combination, may regulate the glutamergic pathway leading to a decreased motor response. Also, it is well accepted that most of the toxic effects of pyrethroid insecticides are mediated through the modification of axonal Na⁺ channels (Narahashi, 1996). Moreover, there is

additional evidence that some of the toxic effects of pyrethroids are mediated by the interaction with GABA receptor-ionophore complex (Crofton and Reiter, 1987; Gammon and Sander, 1985; Lawrence *et al.*, 1985). However, no clear association between the modification of Na⁺ channels and development of sensorimotor deficit has yet been established.

In summary, our results suggest that exposure to physiologically relevant doses of PB, DEET, and permethrin, alone or in combination, lead to sensorimotor deficits and alteration in the cholinergic system in rats. These results further suggest that exposure with these chemicals, alone or in combination, may have played a role in the development of long-term health consequences associated with the PGW veterans. The contribution of cholinergic changes to the behavioral deficit following treatment with these chemicals is not clear at the moment, as these changes may involve a combination of mechanisms related to central and peripheral or neuromuscular system. In a recent study, Nostrandt *et al.*, (1997) observed an insignificant correlation between changes in muscarinic receptor and AChE in the CNS following treatment with chlorpyrifos, which is a more potent cholinotoxic than the chemicals we used in the current studies. However, the possibility remains that the behavioral impairment observed in our studies may also have been a consequence of other generalized abnormalities such as deficit in cognition and motivation because of the changes in cholinergic system. Further studies are in progress to evaluate the histopathological correlates of these behavioral changes.

ACKNOWLEDGMENTS

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**EFFECTS OF DAILY DERMAL APPLICATION OF DEET
AND PERMETHRIN, ALONE AND IN COMBINATION,
ON SENSORIMOTOR PERFORMANCE, BLOOD-BRAIN BARRIER,
AND BLOOD-TESTIS BARRIER IN RATS**

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DEET and permethrin were implicated in the development of illnesses in some veterans of the Persian Gulf War. This study was designed to investigate the effects of daily dermal application of these chemicals, alone or in combination, on the permeability of the blood-brain barrier (BBB) and blood-testes barrier (BTB) and on sensorimotor performance in male Sprague-Dawley rats. Groups of five rats were treated with a dermal daily dose of 4, 40, or 400 mg/kg DEET in ethanol or 0.013, 0.13, or 1.3 mg/kg permethrin in ethanol for 60 d. A group of 10 rats received a daily dermal dose of ethanol and served as controls. BBB permeability was assessed by injection of an iv dose of the quaternary ammonium compound [³H]hexamethonium iodide. While permethrin produced no effect on BBB permeability, DEET alone caused a decrease in BBB permeability in brainstem. A combination of DEET and permethrin significantly decreased the BBB permeability in the cortex. BTB permeability was decreased by treatment with DEET alone and in combination with permethrin. The same animals

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underwent a battery of functional behavior tests 30, 45, and 60 d after exposure to evaluate their sensorimotor abilities. All treatments caused a significant decline in sensorimotor performance in a dose- and time-dependent manner. These results show that daily dermal exposure to DEET, alone or in combination with permethrin, decreased BBB permeability in certain brain regions, and impaired sensorimotor performance.

The insect repellent *N,N*-diethyl-*m*-toluamide (DEET) and the insecticide pyrethroid permethrin [3-phenoxybenzyl-(\pm)-*cis,trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate have been used extensively by humans since their introduction. DEET is commonly used as an effective repellent against mosquitoes, flies, ticks, and other insects in the form of lotion, stick, or spray (Robbins & Cherniack, 1986; McConnell et al., 1986). Extensive and repeated topical application of DEET can result in human and animal poisoning including death (Gryboski et al., 1961; Roland et al., 1985; Edwards & Johnson, 1987; McCain et al., 1997). The symptoms associated with DEET poisoning are characterized by tremors, restlessness, difficulty with speech, seizures, impairment of cognitive function, and coma (McConnell et al., 1986). High levels of DEET exposure have been reported to cause spongiform myelinopathy (Verschoyle & Aldridge, 1990). Because DEET efficiently crosses the dermal barrier (Windheuser et al., 1982; Hussain & Ritschel, 1988; Stinecipher & Shah, 1997) and localizes in dermal fat deposits (Blomquist & Thorsell, 1977; Snodgrass et al., 1982), it is possible that DEET could enhance the availability of drugs and toxicants to other organs, including the brain (Stinecipher & Shah, 1997).

Permethrin is a type I synthetic pyrethroid insecticide that exists in four different stereoisomers (Casida et al., 1983). It provides insecticidal activity for several weeks following a single application. Permethrin toxicity is due to prolonged opening of the sodium channels, leading to repetitive discharges after a single stimulus (Narahashi, 1985). This repetitive nerve action is associated with tremors, hyperactivity, ataxia, convulsions, and, in some cases, paralysis.

During the Persian Gulf War, some service personnel were exposed to a variety of chemicals, including DEET and permethrin (Institute of Medicine, 1995; Abou-Donia et al., 1996). The reported exposure to DEET and permethrin for Gulf War veterans could have occurred during their deployment because these chemicals were used as protective insecticide. Some veterans have reported chronic symptoms including headache, loss of memory, fatigue, muscle and joint pain, and ataxia. All of these symptoms involve either central or peripheral nervous systems function. In these experiments, we studied the effects of DEET and permethrin alone or in combination on blood-brain barrier (BBB) and blood-testes barrier (BTB) permeability and sensorimotor functions following daily dermal application.

MATERIALS AND METHODS

Chemicals

Technical-grade (93.6%) permethrin (\pm)-*cis/trans*-3-(2,2-dichloroethyl)-2,2-dimethylcyclopropane carboxylic acid (3-phenoxyphenyl) methyl ester was obtained from Roussel Uelaf Corp., Pasadena, TX. DEET (97.7%, *N,N*-diethyl-*m*-toluamide), acetylthiocholine iodide, and butyrylthiocholine iodide were purchased from Sigma Chemical Co., St. Louis, MO. [^3H]-Hexamethonium iodide, specific activity 18 Ci/mmol, was obtained from USAMRID, Aberdeen Proving Ground, MD. All other chemicals and reagents were of the highest purity available from commercial sources.

Animals

Male Sprague-Dawley rats (200–250 g) obtained from Zivic Miller, Allison Park, PA, were used. Animals were randomly assigned to control and treatment groups and housed at 21–23°C with a 12-h light/dark cycle. They were supplied with Purina certified rodent chow (Ralston Purina Co., St. Louis, MO) and tap water ad libitum. The rats were allowed to adjust to their environment for a week before treatment. Animal care was in accordance with institutional guidelines.

Treatment

For dermal application of the chemicals, 1 in² of the back of the neck was shaved. The chemicals were applied on the shaved area to give the desired concentration of test compounds in 0.2 ml vehicle. Groups of 10 rats received a daily topical dose of 4, 40, or 400 mg/kg DEET (0.1 \times , 1 \times , and 10 \times dose) in 70% ethanol or 0.013, 0.13, or 1.3 mg/kg (0.1 \times , 1 \times , and 10 \times dose) permethrin in 70% ethanol, alone or in combination. Control animals received an equal volume of the vehicle. The treatment was carried out daily, 7 d/wk, for 60 d. The 1 \times dose of DEET and that of permethrin are based on an estimate of exposure that may have occurred to army personnel during Gulf War. For combined exposure, each chemical was given at 0.1 \times , 1 \times , or 10 \times concentration.

For BBB studies, 24 h after the last treatment, subgroups of 5 animals were anesthetized and then injected in the tail vein under anesthesia with [^3H]hexamethonium iodide (10 μCi , mixed with cold hexamethonium iodide) to give a final dose of 0.7 mg/kg (1 $\mu\text{Ci/kg}$). After 10 min, animals were anesthetized with ketamine/xylazine, blood was collected from the heart with heparinized syringes, and the animals were sacrificed by decapitation. Brains and testes were removed and placed in ice-cold normal saline. Brain regions were dissected on ice into cortex, brainstem, mid-brain, and cerebellum. Following dissection, the brain regions and testes were snap frozen in liquid nitrogen. Plasma was separated from whole blood by centrifugation. Plasma, brain regions, and testes were stored at –20°C for later analysis.

For the determination of [^3H]hexamethonium iodide uptake in tissues and plasma, a weighed amount was subjected to oxygen combustion using a Packard 306B tissue oxidizer (Packard Instrument Co., Downers Grove, IL). Total radioactivity present in tissues and plasma was determined in triplicate in a Beckman LS-6500 multipurpose scintillation spectrometer (Beckman Instruments Corp., Palo Alto, CA). The radioactivity was calculated as disintegrations per minute (dpm) per gram tissue divided by dpm per milliliter plasma for each animal.

Statistical Analysis

The data from BBB experiments were analyzed by analysis of variance (ANOVA) for the test of significance. The criterion for significance was set at $p < .05$. The graphs were generated on Excel graphics for Macintosh and are presented as mean \pm SE of percent of control.

Behavioral Studies

A battery of standardized tests was employed. These behavioral tests were designed to measure sensorimotor reflexes, motor strength, and coordinated gait (Bederson et al., 1986; Markgraf et al., 1992; Goldstein, 1993). All behavioral testing was performed by trained observers blind to the animals' treatment status and was carried out in a soundproof room with subdued lighting (less than 10.76 lumen/m², ambient light). Rats were handled for 2 min daily for 5 d during the week prior to behavioral testing.

Postural Reflexes

Description Rats are held gently by the tail, 1 m above the floor, and were observed for forelimb extension. Normal rats extend both forelimbs. Consistent flexion of the forelimb is an abnormal response. Rats with consistent forelimb flexion are then further assessed by placing each on a large sheet of plastic-coated paper that can be gripped with the forepaws. With the tail held, gentle lateral pressure is applied behind the rat's shoulder until the forelimb slides several inches. The maneuver is repeated five times in each direction. Normal rats resist lateral pressure by gripping the coated paper.

Scoring Grade 0 was given to rats without evidence of consistent forelimb flexion when held above the floor; grade 1, to rats with consistent forelimb flexion; and grade 2, to otherwise grade 1 rats that do not resist lateral pressure on at least three of five trials in either direction.

Limb Placing

Description Visual, tactile, and proprioceptive forelimb placing responses were examined. For visual placing, rats are held in the examiners' hands 10 cm above the tabletop with forelimbs hanging free. The rats are then slowly tilted toward the table. Intact rats reach toward the table with both forepaws. For tactile placing, the dorsal and then lateral portions of

the forepaws are touched to the table edge. Intact rats immediately place the paw on the surface of the table. Proprioceptive placing is tested by pushing the forepaw onto the table edge. Care is taken to avoid the vibrissae touching the table.

Scoring For each test, grade 0 is assigned if the placing response is immediate; grade 1, if the placing response is slow or delayed; and grade 2, if the placing response does not occur within 2 s.

Orienting to Vibrissae Touch

Description The rat is placed atop an inverted polycarbonate cage and allowed 1 min for habituation. Its vibrissae are then touched with a cotton tipped swab.

Scoring Grade 0 is assigned if the rat orients to the side of the probe on at least two of three trials from each side, and grade 1 if the rat fails to orient on at least two of three trials on either side.

Grip Time

Rats' forepaw grip time was assessed by having them hang from a 5-mm-diameter wood dowel gripped with both forepaws. Time to release their grip was recorded in seconds.

Beam Walking and Beam Score

Description The testing apparatus is a 2.5 × 122-cm wooden beam elevated 75.5-cm above the floor with wooden supports. A 20 × 25 × 24-cm goal box with a 9.5-cm opening is located at one end of the beam. A switch-activated source of bright light (75-W tungsten bulb) and a source of white noise (41 dB at 8000 Hz, 58 dB at 4000 Hz, 56 dB at 2000 Hz, 56 dB at 1000 Hz, 58 dB at 500 Hz, and 52 dB at 250 Hz SPL at the center of the frequency at each octave band) were located at the start end of the beam and served as avoidance stimuli. The rats are first trained to traverse the beam with a series of three approximate trials (i.e., rats are first placed at the entrance to the goal box, then at the mid portion of the beam, and finally at the start end of the beam). Rats are readily trained to perform the beam-walking task (Goldstein, 1993). For the testing trials, the rat was placed at the start end of the beam, near the sources of light and noise.

Scoring Beam-walking ability is measured with a seven-point scoring system scale as previously described (Goldstein, 1993): 1, the rat is unable to place the affected hind paw on the horizontal surface of the beam; 2, the rat places the affected hind paw on the horizontal surface of the beam and maintains balance for at least 5 s; 3, the rat traverses the beam while dragging the affected hind paw; 4, the rat traverses the beam and at least once places the affected hind paw on the horizontal surface of the beam; 5, the rat crosses the beam and places the affected hind limb on the horizontal surface of the beam to aid less than half its steps; 6, the

rat uses the affected hindpaw to aid more than half its steps; and 7, the rat traverses the beam with no more than two footslips. In addition, the latency until the animal's nose enters the goal box (up to 90 s) is recorded for the final trial. Rats that fall off the beam are assigned latencies of 90 s.

Inclined Plane

The rats were placed on a flat plane in the horizontal position, with the head facing the side of the board to be raised, as described by Yonemori et al. (1998). The board was slowly rotated to the vertical position. Two trials were performed for each testing session.

Scoring The angle at which the rat begins to slip downward was recorded. The results of two trials were averaged at each time point.

Statistical Analysis

For continuous data, groups were compared by two-way repeated-measures ANOVA. The significance of post hoc pairwise comparison was determined with Fisher's least significant difference (LSD) tests. If indicated for nonparametric data, comparisons across treatment groups were made with the Kruskal-Wallis test.

RESULTS

General Observations

The clinical condition of animals treated with daily dermal application of 4, 40, or 400 mg/kg DEET in ethanol and 0.013, 0.13, or 1.3 mg/kg permethrin in ethanol alone or in combination was not different from controls. No difference was also observed in the weights of treated animals as compared with control.

Blood-Brain Barrier and Blood-Testis Barrier Permeability

Effects of 60 daily dermal applications of DEET and permethrin, alone and in combination, at 3 dose levels (0.1×, 1×, and 10×), on BBB integrity are presented in Figures 1–3 and are given as percent of control of the mean of the ratio between brain and plasma [³H]hexamethonium iodide uptake. A decreased uptake of [³H]hexamethonium iodide was observed in the brainstem of the animals treated with DEET at 1× and 10× dose when compared with the control group (Figure 1). Brainstem was the most affected by DEET treatment, with a decrease of 78, 66, and 65% observed at 0.1×, 1×, and 10× doses, respectively. DEET treatment alone also caused a decrease in BTB permeability. Animals treated with permethrin at all the three doses did not demonstrate any significant changes in BBB permeability in the brain or in the testes when compared to control animals (Figure 2). However, animals treated with DEET + permethrin did exhibit a decrease in BBB permeability (Figure 3). The decrease in BBB permeability in the cortex was exacerbated by the combination of DEET

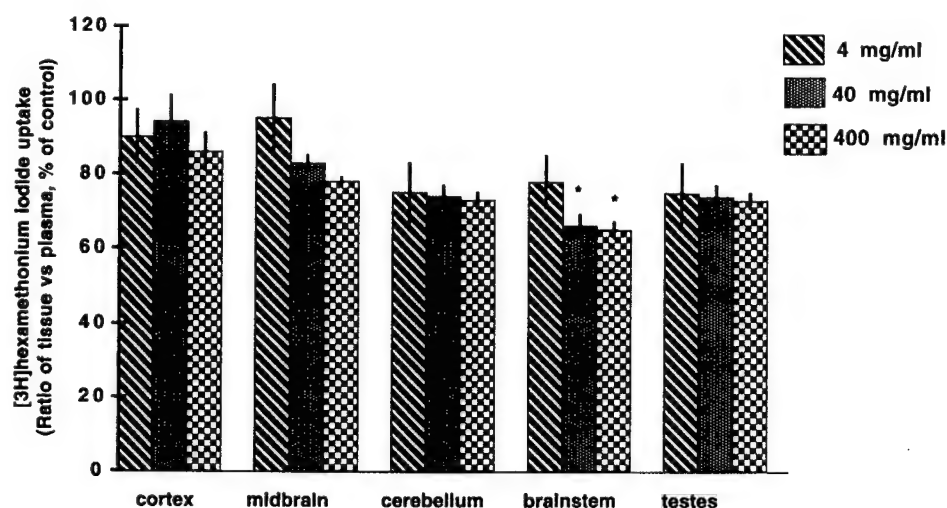


FIGURE 1. [^3H]Hexamethonium iodide uptake in brain regions and testes of the animals treated with DEET. Animals were treated with 60 daily doses of 4 mg/kg, 40 mg/kg, or 400 mg/kg DEET (0.1 \times , 1 \times , and 10 \times dose) by dermal application. [^3H]Hexamethonium iodide was given iv and brain regions were dissected. Radioactivity was measured in each region as described in Materials and Methods. The values represent the ratio between tissue uptake and plasma and are presented as mean \pm SE of percent of control. The control values were: cortex, 0.32 ± 0.05 ; midbrain, 0.48 ± 0.06 ; cerebellum, 0.47 ± 0.09 ; brainstem, 0.45 ± 0.10 ; testes, 0.47 ± 0.09 . Asterisk indicates p value $< .05$.

with permethrin ($p < .02$ at 1 \times and $p < .01$ at 10 \times dose). These animals showed a significant decrease in BBB permeability in the cortex that ranged from 86% to 72% of control.

Behavioral Performance

Each compound was given alone and in combination ($n = 5$ per group) at 1 of 3 doses (0.1 \times , 1 \times , and 10 \times) with a series of behavioral testing performed on d 30, 45, and 60. Figure 4 gives beam walking scores; Figure 5 beam walking times; Figure 6, inclined plane responses; and Figure 7 forepaw grip time for each treatment and for each time point. There was no effect of any of the drugs on postural reflexes, limb placing, or vibrissae touch (data not shown).

For beam walk score (Figure 4), control animals consistently showed completely normal performance aside from the 30-d testing in the permethrin experiment. The graphs otherwise show a dose response with greatest deficits at increasing dose as well as evidence of progressively declining performance over time for animals that received permethrin alone or a combination of DEET and permethrin. For DEET, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,16} = 17.3$, $p < .0001$) and a significant dose \times time interaction (ANOVA $F_{3,6} = 9.7$, $p < .0001$). For the combination, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,13} = 267.6$, $p < .0001$) and a signifi-

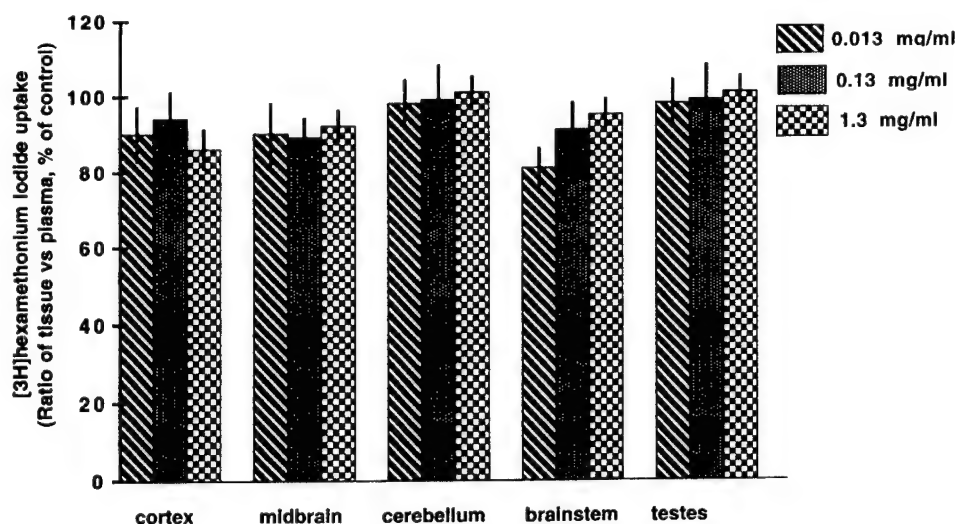


FIGURE 2. [^3H]Hexamethonium iodide uptake in brain regions and testes of the animals treated with permethrin. Animals were treated with 60 daily doses of 0.013 mg/kg, 0.13 mg/kg, or 1.3 mg/kg permethrin (0.1 \times , 1 \times , and 10 \times dose) by dermal application. [^3H]Hexamethonium iodide was given iv, and brain region and testes were dissected. All other details were as described for Figure 1. The values represent the ratio between tissue uptake and plasma and are presented as mean \pm SE of percent of control. The control values were: cortex, 0.26 ± 0.05 ; midbrain, 0.28 ± 0.03 ; cerebellum, 0.24 ± 0.03 ; brainstem, 0.25 ± 0.04 ; testes, 0.24 ± 0.02 .

cant dose \times time interaction (ANOVA $F_{3,6} = 3.9$, $p = .0047$). At the 30-d time point, the 0.1 \times dose of DEET ($p < .0001$) but neither permethrin nor the combination differed from controls.

For beam walk time (Figure 5), there were also significant relationships between each compound and performance. For DEET, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,16} = 144.3$, $p < .0001$) but no dose \times time interaction (ANOVA $F_{3,6} = 1.94$, $p = .2457$). For permethrin, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,16} = 1.78$, $p = .0001$) and a significant dose \times time interaction (ANOVA $F_{3,6} = 3.74$, $p < .0062$). For the combination, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,16} = 86.0$, $p < .0001$) and a significant dose \times time interaction (ANOVA $F_{3,6} = 10.2$, $p = .0047$). At the 30-d time point, the 0.1 \times dose of DEET ($p < .0001$) but neither permethrin nor the combination differed from controls.

Inclined plane performance tests (Figure 6) showed significant relationships between each compound and performance. For DEET, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,6} = 4.21$, $p < .001$). For permethrin, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,16} = 26.6$, $p < .0001$) and a significant dose \times time interaction (ANOVA $F_{3,6} = 3.74$, $p = .0042$). For the combination, two-way repeated measures ANOVA showed a signifi-

cant effect of dose (ANOVA $F_{3,16} = 24.9$, $p < .0001$) and a significant dose \times time interaction (ANOVA $F_{3,6} = 10.2$, $p = .0035$). At the 30-d time point, the 0.1 \times dose of DEET ($p = .04$), and the combination ($p = .005$) differed from controls. Permethrin did not differ from controls significantly ($p = .06$).

Finally, for forepaw grip test (Figure 7), there was a clear effect of all doses at all time points. For DEET, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,16} = 18.7$, $p = .0060$). For permethrin, two-way repeated-measures ANOVA showed a significant effect of dose (ANOVA $F_{3,16} = 82.1$, $p < .0001$). Because all groups were severely affected even at the first time point, the dose \times time interactions were not significant. At the 30-d time point the 0.1 \times dose of DEET, permethrin, and the combination each differed significantly from controls ($p < .001$, respectively).

The relationships between each test compound and dose in comparison to pooled controls are shown in Figure 8 for the 30-d time point. Declining performance with increasing dose on beam walking is apparent for animals that received DEET or the DEET-permethrin combination (top panels); poorer inclined plane responses with increasing dose are present for animals that received permethrin or the DEET-permethrin combination (bottom left panel); and all animals at all doses had impaired paw grip strength (bottom right panel).

A comparison of the significance at the lowest and highest dose of each agent compared to the combination at the 30-d time point for

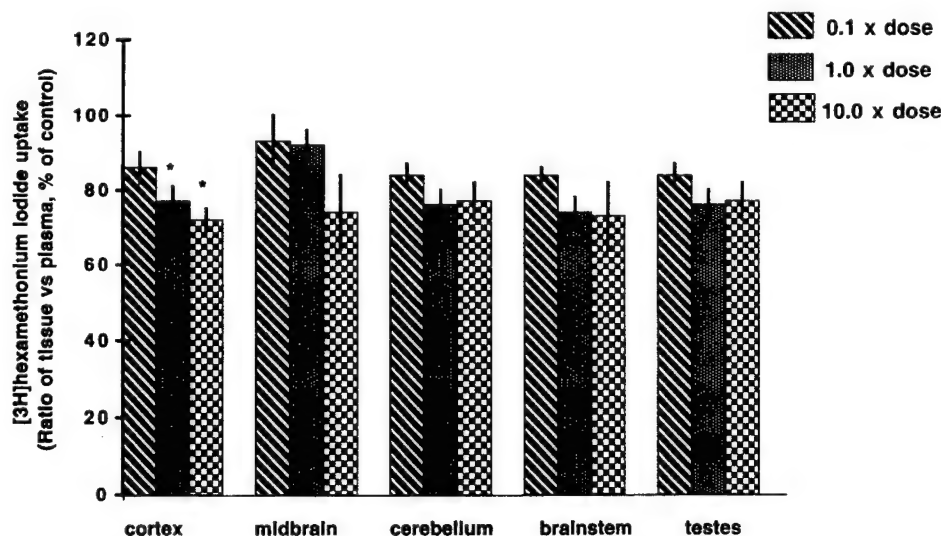


FIGURE 3. $[^3\text{H}]$ Hexamethonium iodide uptake in brain region and testes of the animals treated with DEET and permethrin together by dermal application for 60 d. All other details were as described for Figure 1. The values represent the ratio between tissue uptake and plasma and are presented as mean \pm SE of percent of control. The control values were: cortex, 0.41 ± 0.03 ; midbrain, 0.49 ± 0.03 ; cerebellum, 0.42 ± 0.02 ; brainstem, 0.40 ± 0.03 ; testes, 0.42 ± 0.03 . Asterisk indicates p value $< .05$.

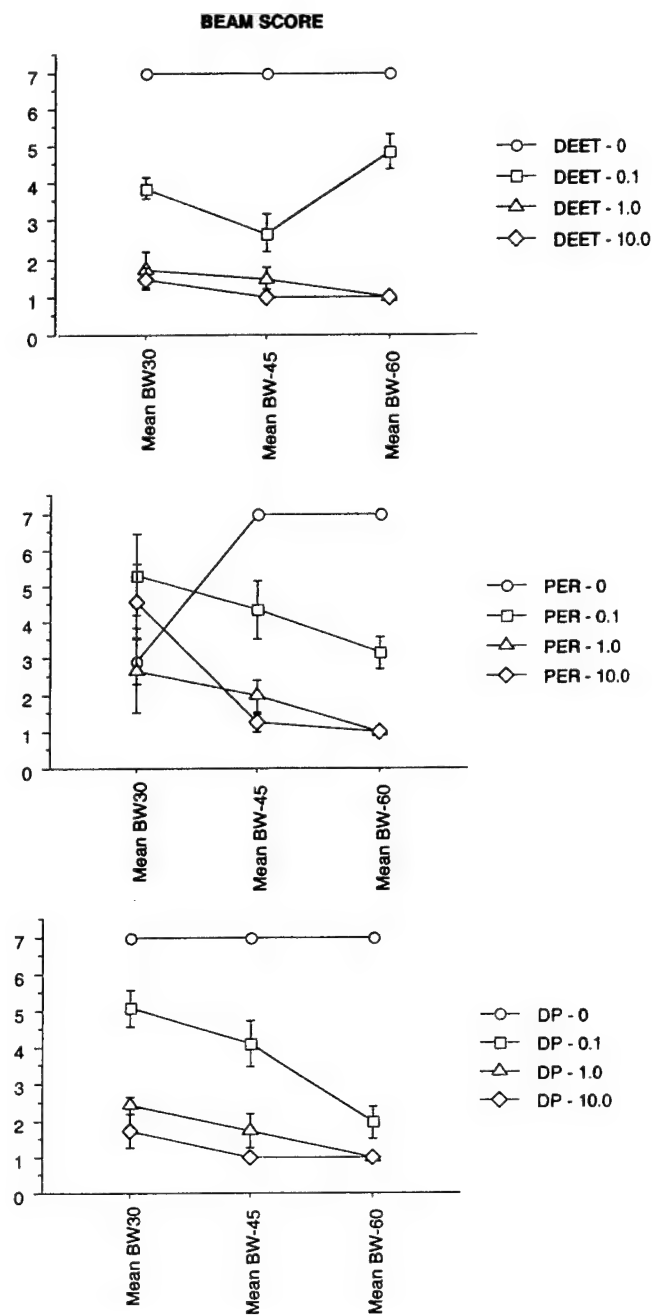


FIGURE 4. Beam score response of animals treated with DEET or permethrin or a combination of DEET and permethrin for 60 d by dermal application. The doses are represented as 0.1 \times , 1 \times , or 10 \times of real-life exposure, which is equivalent to 4, 40, and 400 mg/kg for DEET and 0.013, 0.13, and 1.3 mg/kg of permethrin, respectively. The animals were examined on d 30, 45, and 60, as presented on the x axis. The y axis presents the mean of the beam score. Evaluations were carried out as described in Materials and Methods.

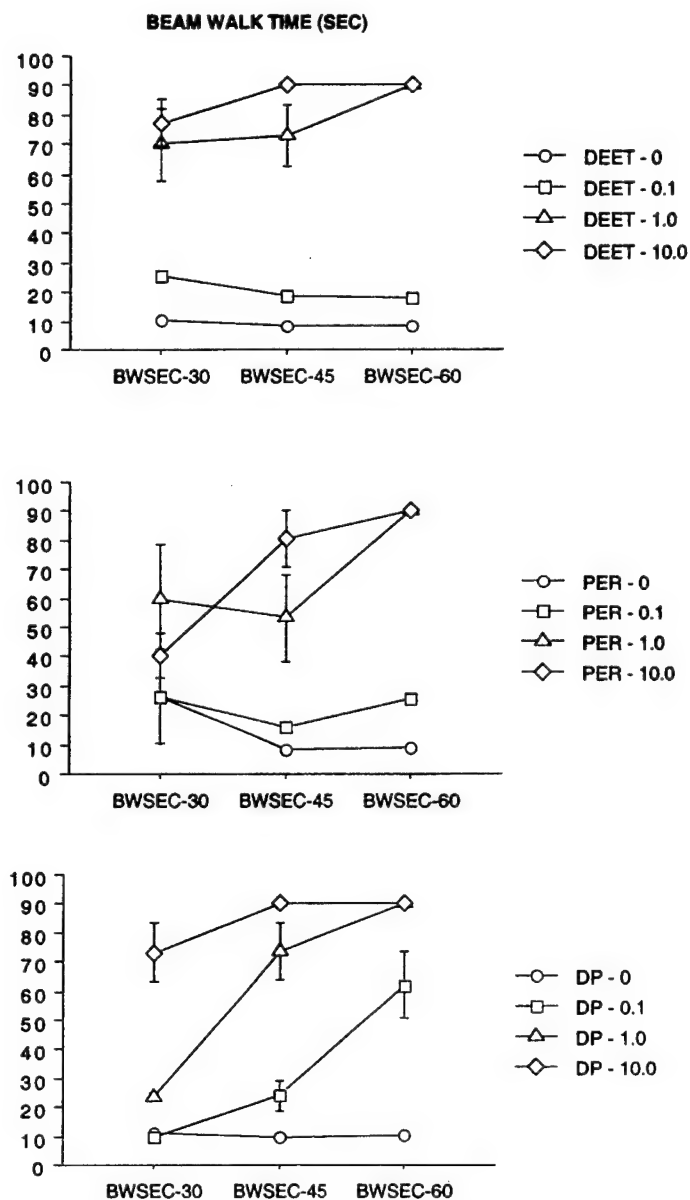


FIGURE 5. Beam walk time in seconds of the animals treated with DEET or permethrin or a combination of DEET and permethrin for 60 d by dermal application. All the other details were as described for Figure 4.

each behavioral parameter is presented in Table 1. There were significant differences between the highest dose in combination and permethrin alone in beam walk score and beam walk time, and between the highest dose of the combination and DEET for inclined plane performance.

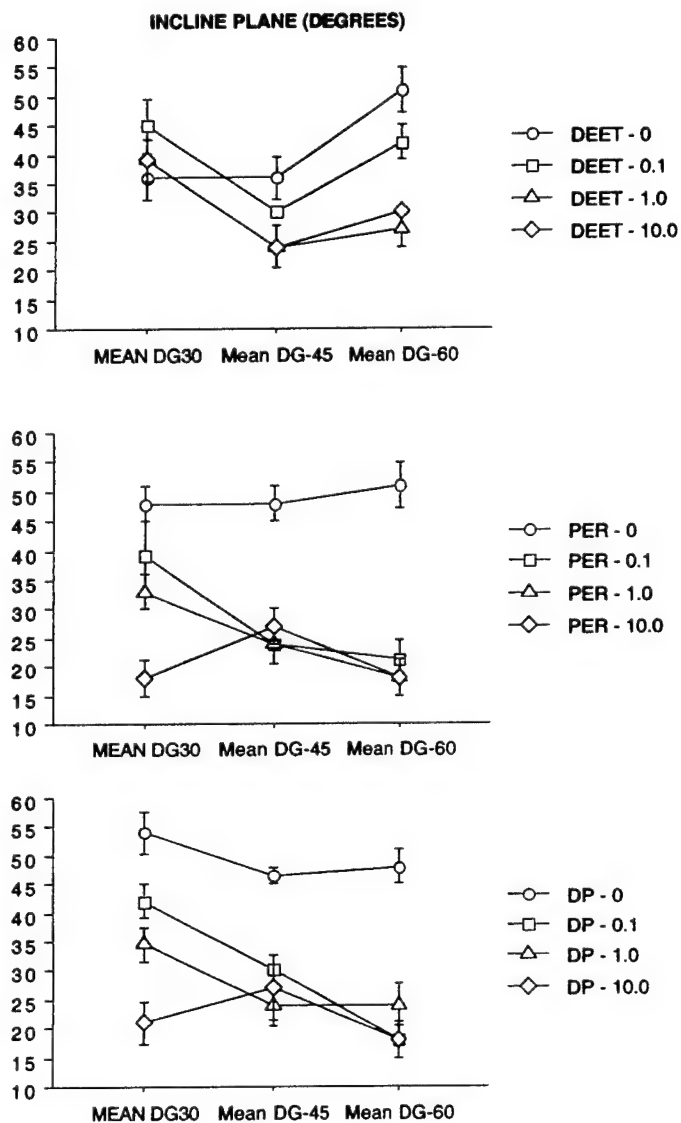


FIGURE 6. Incline plane in degrees of the animals treated with DEET or permethrin or a combination of DEET and permethrin together for 60 d by dermal application. The data on the y axis represents the mean of the angles in degrees at which animal falls off the plane as described. All other details were as described for Figure 4.

In summary, these studies demonstrate impaired behavioral performances at even the lowest administered dose of each agent for at least some sensorimotor parameters. The combination of the two agents resulted in significantly poorer performance than either agent alone, but only for some behavioral parameters and only at high dose.

DISCUSSION

This study was designed to investigate the effects of repeated daily dermal application of DEET and permethrin alone or in combination on sensorimotor function and the integrity of the BBB in male Sprague-Dawley rats. The route of exposure and the dose levels of test compounds were

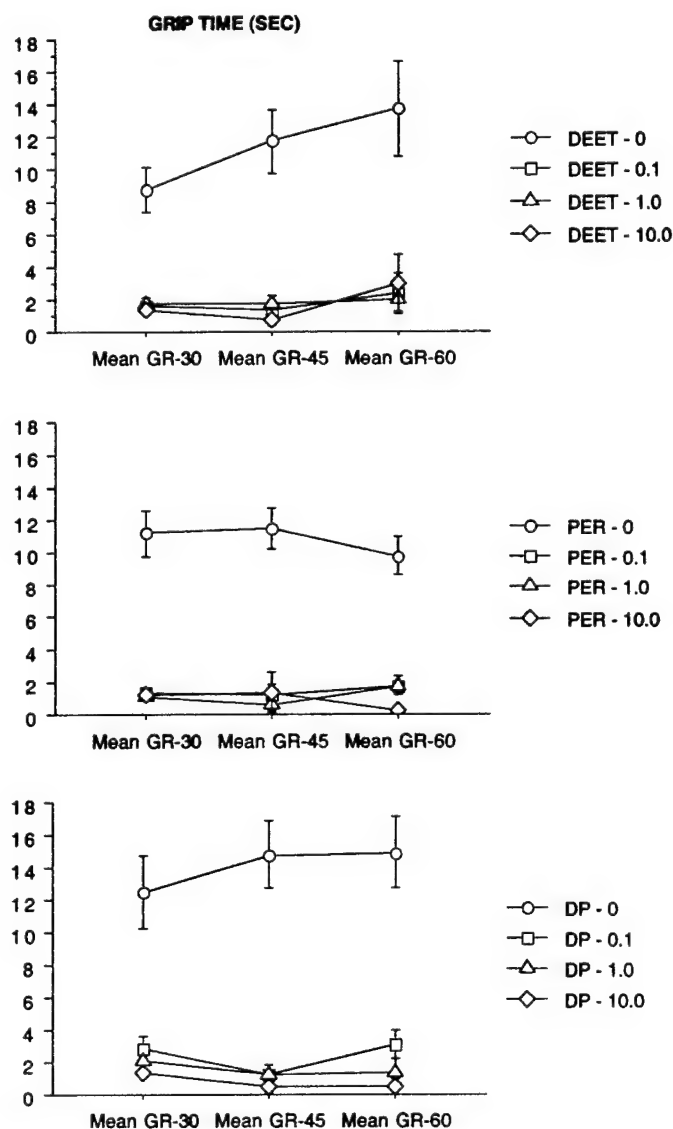


FIGURE 7. Grip time in seconds of the animals treated in the DEET or permethrin or DEET and permethrin in combination for 60 d by dermal application. Numbers on the y axis represents the time in seconds the animals are able to hang from a wooden dowel gripped with both forepaws. The x axis represents the days when the evaluation was carried out.

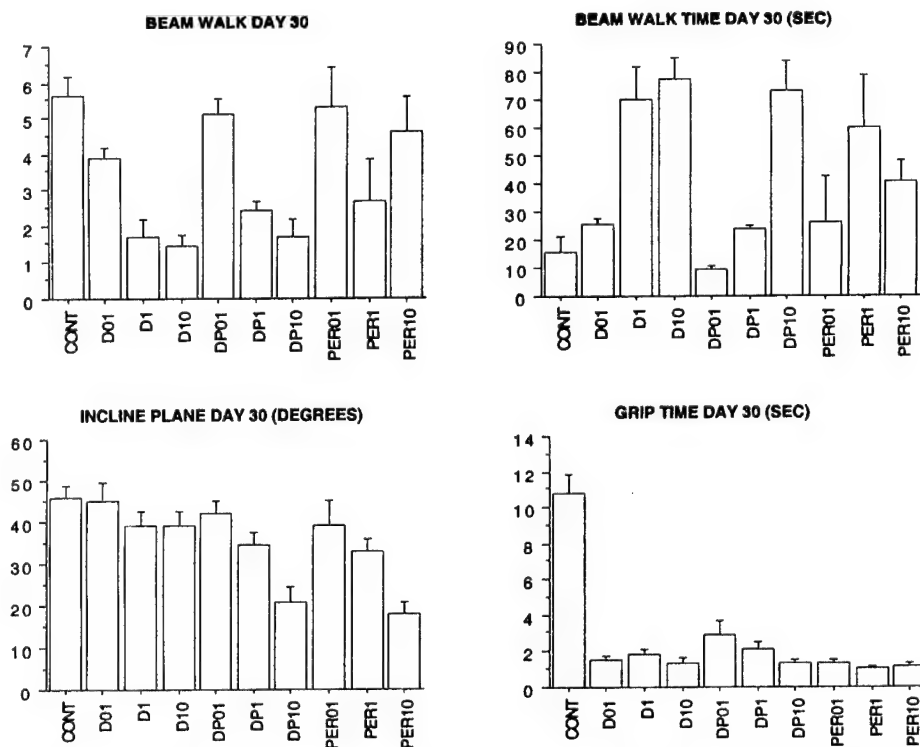


FIGURE 8. Presentation of the data as bar graph for the d 30 observation on the effects of DEET or permethrin or a combination of DEET and permethrin on behavioral performance.

chosen to closely reflect those present during the Gulf War. Both test compounds were applied dermally using 0.1, 1, and 10 times the estimated real life doses of 40 mg/kg/d for DEET and 0.13 mg/kg/d for permethrin. Our data suggest that physiologically relevant exposure to DEET for 60 d caused a decrease in BBB permeability in the brainstem at 1× and 10× doses. Permethrin exposure alone had no observable effect on the BBB permeability. However, a combination of the two chemicals caused a decrease in the BBB permeability in the cortex in a dose-dependent fashion as compared to DEET or permethrin alone, suggesting that the two chemicals together may have additive effects, but only in selected brain region(s) such as cortex. In our earlier studies in hens we reported that coexposure with pyridostigmine bromide, DEET, and permethrin resulted in a higher level of toxicity than each chemical alone (Abou-Donia et al., 1996). In those studies we used a subcutaneous route of exposure at relatively higher doses of each chemical. These results suggest that DEET exposure alone or in combination by dermal application can lead to BBB permeability changes in certain brain region(s) that can have important physiological/pharmacological consequences.

The BBB regulates the entry of molecules into the central nervous sys-

tem (CNS) based on the size, charge, hydrophobicity, and/or affinity of carriers. The selective nature of the BBB helps maintain the homeostasis of the CNS environment to ensure proper brain function (Joo, 1996). The BBB consists of the cerebral capillary endothelium, which contains tight junctions. These tight junctions form rows of extensive overlapping occlusions that block the intercellular route of solute entry into the CNS. Any changes in the basal permeability of the cerebral capillary endothelium can exacerbate a variety of pathological processes. Our finding that DEET exposure alone or in combination with permethrin caused a decrease in BBB permeability could restrict passage of important molecules that are required for normal homeostasis in the CNS. Our studies do not provide any data on the levels of DEET or permethrin in the CNS. A recent *in vitro* study (Baynes et al., 1997) suggested that in coexposure with DEET and permethrin, DEET antagonizes the absorption of permethrin in different model systems of cutaneous exposure. The decrease in BBB permeability with combined exposure does not necessarily reflect an antagonistic effect. It could also mean that both chemicals in combination cause changes in cerebrovascular endothelium, leading to a decrease in [^3H]hexamethonium iodide uptake. The decrease in BBB permeability could be mediated by one of several mechanisms; for example, it is known that membrane fluidity changes induced by ethanol cause a decrease in the passage of [^3H]vincristine into the CNS (Domer & Smith, 1988). A decrease in blood flow to the CNS could also cause a reduction in the availability of [^3H]hexamethonium iodide to the CNS.

TABLE 1. Comparison of Lowest and Highest Dose of Each Agent or Combination at 30 d

Test	Dose	Comparison	<i>p</i> ^a
Beam-walk score	Low	Comb vs. Per	NS
	High		<.02
	Low	Comb vs. DEET	NS
	High		NS
Beam-walk time	Low	Comb vs. Per	NS
	High		<.03
	Low	Comb vs. DEET	NS
	High		NS
Incline plane	Low	Comb vs. Per	NS
	High		NS
	Low	Comb vs. DEET	NS
	High		<.004
Paw grip	Low	Comb vs. Per	NS
	High		NS
	Low	Comb vs. DEET	NS
	High		NS

^aFisher LSD.

Cerebrovascular endothelium is known to express multidrug transporting *p*-glycoprotein (*p*-gp) at BBB sites (Gottesman & Pastan, 1993). It has been proposed that *p*-gp localization at the BBB serves to protect the CNS by causing efflux of drugs and chemicals. Indeed, deletion of the *p*-gp gene has been shown to lead to massive localization of chemotherapeutic changes in the brain, suggesting that *p*-gp plays an important role in efflux of drugs and chemicals (Schinkel et al., 1994). Therefore, it is possible that under our experimental conditions, DEET alone or in combination with permethrin may regulate the expression of *p*-gp. The neurotoxic effects of DEET may be augmented both by its increased localization into the CNS because of its lipophilicity, and because of decrease in the transport of otherwise critical molecules. It has also been reported that elevated cAMP levels decrease the BBB permeability in rat pial vessels and frog peripheral capillaries (He & Curry, 1993). Increased cAMP levels have been shown to reduce BBB permeability induced by cerebral ischemia (Belayev et al., 1998). It is possible that DEET could modulate the levels of cAMP after prolonged exposure. Neurotoxic chemicals induce a hypothermic response, which is known to reduce BBB permeability (Gordon, 1993). Prolonged exposure to DEET may cause a hypothermic response that may be responsible for BBB permeability changes. Tight gap junctions at the BBB are mediated by adherens (Rubin & Staddon, 1999). These are a class of membrane proteins that are critical for maintenance and functioning of BBB. DEET access to the microvascular endothelium could regulate the expression of such junction proteins, resulting in diminished blood flow, thereby reducing the entry of [³H]hexamethonium iodide molecules. Decrease in the BBB permeability by DEET alone or in combination with permethrin could result by any or all of the mechanisms already discussed. It should be noted, however, that the changes observed in BBB permeability are subtle; therefore, additional approaches such as histopathological evaluations may provide definitive proof of the changes in the CNS occurring as a consequence of DEET treatment alone or in combination with permethrin.

Severe CNS toxicity due to DEET and permethrin are apparent only at high doses; for example, DEET-induced signs of CNS depression, death, and protracted seizure activity were observed at several dose levels in rats (Verschoyle et al., 1992). Similar complications have been observed in DEET poisoning in humans (Pronczuk de Garbino & Laborde, 1983; McConnell et al., 1986). Symptoms such as daytime sleepiness and impaired cognitive functions have been shown to result from heavy DEET exposure, whereas gait balance and dexterity were moderately affected (McConnell et al., 1986). Additionally, lethargy has been noted as a prominent feature in severe acute DEET intoxication (Tenebein, 1987; Snyder et al., 1986). A relatively recent study found a decrease in motor activity in male and female rats after a single dose DEET treatment (Verschoyle et al., 1992). Permethrin-induced behavioral changes have also been documented in animals (Hoy et al., 2000). Permethrin-induced neurotoxic changes

are characterized by aggressive sparring, increased sensitivity to external stimuli, and fine tremors that progress to whole-body tremors and prostration (Verschoyle & Barnes, 1972; Verschoyle & Aldridge, 1980; Bradbury & Coats, 1989). McDaniel and Moser (1993) reported a decrease in grip strength and induced head and forelimb shaking. Additionally, decreased operant response rate, deficit in role mode performance, and a decrease in turning-wheel activity have been observed (Bloom et al., 1983; Glowa, 1986). Studies by Crofton and Reiter (1988) have shown a decrease in locomotor activity in rats exposed to permethrin. Most of these reported studies used routes of exposure that may not be directly applicable to contact exposure, as is believed to have occurred during the Gulf War. Our data suggest that dermal exposure with these chemicals for a long period could potentially cause changes to the BBB that may cause pathological changes in the CNS.

Our data on sensorimotor function are consistent with our previous work (Abou-Donia et al., 1996). There was no effect of any of the drugs, alone or in combination, on simple sensorimotor reflexes. However, our studies suggest that DEET and permethrin alone or in combination cause a deficit in sensorimotor performance even at one-tenth the real-life exposure dose. Elevating dose and longer duration of exposure caused increasingly greater deficits. The biochemical and cellular bases of these effects are not clearly understood. The behavioral effects of these agents may be mediated centrally, peripherally, or through a combination of both mechanisms. The lack of a consistent difference between each dose given alone or in combination suggests at least a partial peripheral mechanism of action, given our data on the effects of these agents on the BBB. Future studies on histopathological damage by these agents and their association with specific behavioral changes will provide a mechanistic explanation for the behavioral changes observed in these studies.

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Subchronic Dermal Application of *N,N*-Diethyl *m*-Toluamide (DEET) and Permethrin to Adult Rats, Alone or in Combination, Causes Diffuse Neuronal Cell Death and Cytoskeletal Abnormalities in the Cerebral Cortex and the Hippocampus, and Purkinje Neuron Loss in the Cerebellum

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N,N-Diethyl *m*-toluamide (DEET) and permethrin have been implicated as potential neurotoxic agents that may have played an important role in the development of illnesses in some veterans of the Persian Gulf War. To determine the effect of subchronic dermal application of these chemicals on the adult brain, we evaluated histopathological alterations in the brain of adult male rats following a daily dermal dose of DEET (40 mg/kg in 70% ethanol) or permethrin (0.13 mg/kg in 70% ethanol) or a combination of the two for 60 days. Control rats received a daily dermal dose of 70% ethanol for 60 days. Animals were perfused and brains were processed for morphological and histopathological analyses following the above regimen. Quantification of the density of healthy (or surviving) neurons in the motor cerebral cortex, the dentate gyrus, the CA1 and CA3 subfields of the hippocampus, and the cerebellum revealed significant reductions in all three treated groups compared with the control group. Further, animals receiving either DEET or permethrin exhibited a significant number of degenerating (eosinophilic) neurons in the above brain regions. However, degenerating neurons were infrequent in animals receiving both DEET and permethrin, suggesting that neuronal cell death occurs earlier in animals receiving combined DEET and permethrin than in animals receiving either DEET or permethrin alone. The extent of neuron loss in different brain regions was similar among the three treatment groups except the dentate gyrus, where neurodegeneration was significantly greater with exposure to DEET alone. The neuron loss in the motor cerebral cortex and the CA1

subfield of all treated groups was also corroborated by a significant decrease in microtubule associated protein 2-immunoreactive elements (15–52% reduction), with maximal reductions occurring in rats receiving DEET alone; further, the surviving neurons in animals receiving both DEET and permethrin exhibited wavy and beaded dendrites. Analysis of glial fibrillary acidic protein immunoreactivity revealed significant hypertrophy of astrocytes in the hippocampus and the cerebellum of all treated groups (24–106% increase). Thus, subchronic dermal application of DEET and permethrin to adult rats, alone or in combination, leads to a diffuse neuronal cell death in the cerebral cortex, the hippocampal formation, and the cerebellum. Collectively, the above alterations can lead to many physiological, pharmacological, and behavioral abnormalities, particularly motor deficits and learning and memory dysfunction. © 2001 Academic Press

Key Words: brain injury; Gulf War syndrome; glial hypertrophy; glial fibrillary acidic protein; microtubule associated protein 2; neuron degeneration.

INTRODUCTION

During the Persian Gulf War (PGW) in 1991, many service personnel were exposed to a variety of chemicals, including *N,N*-diethyl *m*-toluamide (DEET) and permethrin (2, 18). DEET and permethrin, in particular, were used extensively by service personnel as a protection against insectborne diseases (18). In the last decade, many veterans have complained of chronic symptoms including headache, loss of memory, fatigue, muscle and joint pain, and ataxia. DEET and permethrin have been implicated as two of the likely neurotoxic agents that may have played a significant role

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in the occurrence of neurological disorders in some veterans of the PGW.

The insect repellent DEET and the pyrethroid insecticide permethrin (3-phenoxybenzyl [\pm]-*cis*, *trans*-3-[2,2-dichlorovinyl]-2-dimethylcyclopropane-1-carboxylate) have been used extensively by humans since their introduction. DEET is used as an effective repellent against mosquitoes, flies, ticks, and other insects in the form of lotion, stick, or spray (21, 28). Extensive and repeated topical application of DEET can result in human and animal poisoning including death (10, 15, 20, 29). The symptoms associated with DEET poisoning are characterized by tremors, restlessness, difficulty with speech, seizures, impairment of cognitive function, and coma (21). High levels of DEET exposure have been reported to cause spongiform myelinopathy (38). DEET efficiently crosses the dermal barrier (17, 34, 40,) and localizes in dermal fat deposits (5, 32). Permethrin is a type I synthetic pyrethroid insecticide that exists in four different stereoisomers (8). It provides insecticidal activity for several weeks following a single application. Permethrin toxicity is due to prolonged opening of the sodium channels, leading to repetitive discharges after a single stimulus (23). This repetitive nerve action is associated with tremors, hyperactivity, ataxia, convulsions, and, in some cases, paralysis (30).

The majority of the symptoms reported by the affected veterans of the PGW involve abnormal regulation of functions in either the central or peripheral nervous system or both. Recent studies in our laboratory have suggested significant sensorimotor deficits and blood-brain barrier disruption following exposure to DEET and permethrin (1). In this study, we evaluated the extent of neurodegeneration within the motor cerebral cortex, the dentate gyrus, the CA1 and CA3 subfields of the hippocampus, and the cerebellum of adult rats after daily dermal application of DEET and permethrin for 60 days, alone or in combination. Dermal doses of 40 mg/kg DEET and 1.3 mg/kg permethrin were applied daily, because they were determined to be the doses that military personnel were exposed to during the Persian Gulf War (Dr. W. C. McCain, U.S. Army Center for Health, Promotion, and Prevention Medicine, Aberdeen Proving Ground, MD, personal communication).

Following exposure of animals to DEET and permethrin for 60 days, alone or in combination, we rigorously quantified neurodegeneration in the above brain regions by several indices. These include: (i) measurement of the density of both healthy (surviving) and dying neurons; (ii) quantification of the reductions in the microtubule-associated protein 2 (MAP-2)-immunoreactive elements; and (iii) measurement of the extent of upregulation in the glial fibrillary acidic protein (GFAP)-immunopositive structures. In addition, we investigated the histopathological alterations in surviv-

ing neurons, particularly the orientation and cytoarchitecture of dendrites using MAP-2 immunostaining.

MATERIALS AND METHODS

Chemicals and Antibodies

DEET (97.7%, *N,N*-diethyl *m*-toluamide) was purchased from Sigma Chemical Company (St. Louis, MO). Technical-grade permethrin, (\pm)-*cis/trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid (3-phenoxyphenyl) methyl ester (93.6%), was obtained from Roussel Uclaf Corporation (Pasadena, TX). The monoclonal antibody (SMI 52) against MAP-2 was from Sternberger Monoclonals (Lutherville, MD), the polyclonal antibody against GFAP was from Dako Laboratories (Carpinteria, CA), and the avidin-biotin complex (ABC) detection kits were purchased from Vector Labs (Burlington, CA). All other chemicals and reagents were of highest purity available from commercial sources.

Animals

Male Sprague-Dawley rats (200–250 g) obtained from Zivic Miller (Allison Park, PA) were used. Animals were randomly assigned to control and treatment groups of five rats ($n = 5$) and housed at 21–23°C with a 12-h light/dark cycle. They were supplied with Purina Certified Rodent Chow (Ralston Purina Co., St. Louis, MO) and tap water ad libitum. The rats were allowed to adjust to their environment for a week before treatment. Animal care was in accordance with the Army Guidelines and Duke University Animals Care and Use Committee.

Dermal Application of DEET and Permethrin

The chemicals (DEET and permethrin) were applied directly to the skin of preclipped areas (2.5 cm²) in the back of the neck to give the desired concentration of test compounds in 1 ml of the vehicle solution. Groups of five rats received a daily dermal dose of 40 mg/kg DEET in 70% ethanol, or 0.13 mg/kg permethrin in 70% ethanol, or the combination of DEET and permethrin. Control animals received an equal volume of the vehicle. The treatment was carried out daily, 7 days a week, for 60 days. The doses of DEET and permethrin are based on an estimate of the exposure that may have occurred to army personnel during the PGW (1). For combined exposure, both chemicals (at the single dose level) were applied simultaneously on adjacent areas of the skin in the back of the neck.

Histopathological Assessment

Twenty-four hours after the last dose, animals belonging to each group ($n = 5$ per group) were anesthetized with pentobarbital (100 mg/kg) and perfused through the heart with saline followed by 4% parafor-

maldehyde and 0.1% glutaraldehyde in Tris buffer. The brains were removed, postfixed, and embedded in paraffin. Four-micrometer-thick coronal sections were cut through different brain regions. In every brain, representative sections ($n = 5$) through the motor and sensory cortex, the septal hippocampus, and the cerebellum were processed and stained with hematoxylin and eosin (H&E) for light microscopy.

MAP-2 and GFAP Immunohistochemistry

Sections were deparaffinized and blocked with 10% normal serum (normal horse serum for MAP-2, normal goat serum for GFAP) in 0.05 M TBS for 30 min. Sections were incubated overnight at room temperature in primary antisera diluted at 1:1000 for MAP-2 in 0.05 M Tris-buffered saline (TBS) containing 1% normal horse serum, and 1:10,000 for GFAP with 0.05 M TBS containing 1% normal goat serum. Following a thorough rinse in 0.05% TBS, sections were incubated for 1 h at room temperature in appropriate biotinylated secondary antibody (i.e., horse anti-mouse IgG for MAP-2, goat anti-rabbit IgG for GFAP, diluted 1:200) containing 1% normal serum (horse serum for MAP-2 staining, goat serum for GFAP staining).

Sections were rinsed with several changes of 0.05 M TBS and incubated for 1 h in the avidin-biotin peroxidase complex solution diluted 1:25 in 0.05 M TBS. Following this, the sections were rinsed with several changes of 0.05 M TBS and incubated with 3,3-diaminobenzene tetrahydrochloride (DAB) for 10 min. The reaction was stopped by several rinses in 0.05 M TBS. The sections were then dehydrated in alcohol, cleared in xylene, and coverslipped with Permount.

Quantitative Evaluation of the Number of Healthy and Dying Neurons in Different Brain Regions

The numerical density of healthy (surviving) and dying neurons per square millimeter of tissue area in H&E-stained sections was measured for layers III and V of the motor cortex, granule cell layer of the dentate gyrus, pyramidal cell layer of CA1 and CA3 subfields of the hippocampus, and Purkinje cell layer of the cerebellum in lobule 2 of the cerebellar vermis and crus 2 ansiform lobule of the cerebellar hemisphere. Five sections through each of the above brain regions were employed for measurements in each animal belonging to the following four groups: (a) control animals ($n = 5$); (b) animals treated with DEET ($n = 5$); (c) animals treated with permethrin ($n = 5$); (d) animals treated with both DEET and permethrin ($n = 5$). Measurements in sections from various groups were performed in a blinded fashion using experimental codes. The coding was such that animal treatments were not known during measuring; however, sections that came from the same animal were identified. All measurements were performed using a Nikon E600 microscope

equipped with eyepiece grid. At a magnification of 400 \times (using 40 \times objective lens and 10 \times eyepieces), both dying and healthy neurons, within a unit area of each section, were counted.

The unit area selected for measurements varied for different regions of the brain, depending on the availability of the overall area for different layers. The area measured was 0.019 mm² for layer III of the motor cortex, 0.063 mm² for layer V of the motor cortex, 0.013 mm² for the dentate granule cell layer, 0.0063 mm² for the CA1 pyramidal cell layer, 0.013 mm² for the CA3 pyramidal cell layer, and 0.0063 mm² for Purkinje cell layer of the cerebellum. For measurement of the surviving neurons, only those that exhibited a hematoxylin-stained nucleus with a clear nucleolus were counted. For measurement of dying neurons, only those neurons that exhibited dense eosinophilic staining in both soma and proximal dendrites were counted. Finally, the density of neurons per unit area was transformed to the numerical density per square millimeter of respective brain region.

The mean value for each of the six brain regions (layers III and V of the motor cortex, granule cell layer of the dentate gyrus, CA1 and CA3 pyramidal cell layers of the hippocampus, and Purkinje cell layer of the cerebellum) was calculated separately for each animal by using data from five sections before the means and standard errors were determined for the total number of animals included per group. Mean values between different groups of animals were compared separately for each of the above brain regions using one-way ANOVA with Student Newman-Keuls multiple comparison post hoc test.

Morphometric Analyses of MAP-2-Positive and GFAP-Positive Immunoreactivity in Different Brain Regions

Morphometric analyses of MAP-2-positive and GFAP-positive immunoreactive structures in different regions were performed by using Scion Image for Windows, based on NIH Image for Macintosh (Scion Corporation, Frederick, MD). For every brain region, two sections were measured in each animal. All data were collected blind to experimental codes and means were calculated for each animal individually before the means were determined for the five animals per group. Statistical comparisons on MAP-2 and GFAP measurements in different brain regions between control and treated groups used ANOVA with Student Newman-Keuls multiple comparison post hoc test.

The area occupied by MAP-2-positive immunoreactive structures per unit area of tissue (0.044 mm²) was determined for layers III and V of the motor cerebral cortex and the CA1 subfield of the hippocampus. The area occupied by GFAP-positive immunoreactive structures per unit area of tissue (0.0176 mm²) was deter-

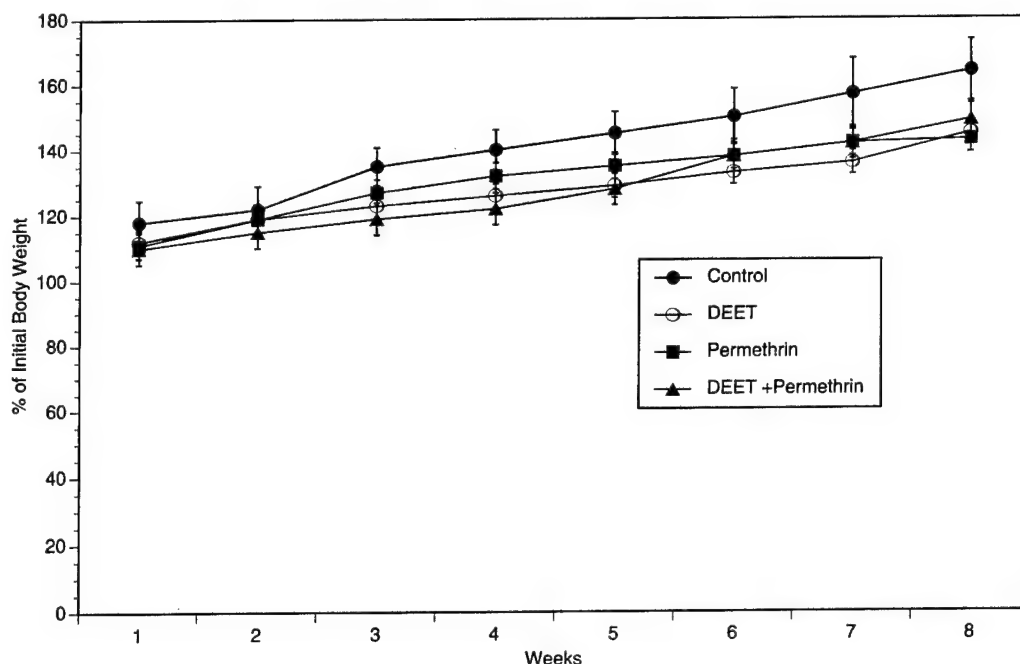


FIG. 1. Effect of daily administration of DEET (40 mg/kg/day, dermal) and permethrin (0.1340 mg/kg/day, dermal) on body weight of rats. Animals were assessed for body weight each week. The percentages of initial body weight for control (mean \pm SEM) are 118 ± 6.7 (Week 1), 122 ± 7.1 (Week 2), 135 ± 5.8 (Week 3), 140 ± 6.1 (Week 4), 145 ± 6.5 (Week 5), 155 ± 8.5 (Week 6), 157 ± 10.7 (Week 7), and 164 ± 9.4 (Week 8). Analysis with one-way ANOVA with Student Newman-Keuls multiple comparisons test revealed no significant differences between groups at all time points during the exposure regimen ($P > 0.1$).

mined in layer V of the motor cerebral cortex, the dentate gyrus, the CA1 and CA3 subfields of the hippocampal formation, and the white matter of the cerebellum. For every region, the microscopic image using 20 \times objective lens was transferred to the computer screen by focusing the appropriate area of the immunostained section with a Nikon E600 microscope equipped with a digital camera (DAGE-MTI, CCD100) connected to an IBM computer. The same intensity of light in the microscope and the same parameters in the digital camera were used to digitize all the samples from different brain regions. Images in the Scion Image are two-dimensional arrays of pixels (picture elements). Pixels are represented by 8-bit unsigned integers, ranging in value from 0 to 255. Scion Image displays zero pixels as white and those with a value of 255 as black. The background and target values were set at 145 and 255, respectively, following digitization of the original gray value image in the computer screen. These values were determined by selecting the background and target areas in several sections from both control and treated animals before commencing the measurements on coded slides for statistical analysis. This scale eliminated the background staining completely and retained all the target (MAP-2- or GFAP-immunopositive) structures in the range (145–255). The binary image of MAP-2- or GFAP-positive elements was then generated by selecting a suitable

threshold value (which varied from 155 to 165) to include all the MAP-2- or GFAP-positive structures without any background. The final binary image was cross-checked with the original gray value image by alternating the two images on the computer screen.

Finally, the image was frozen and the area occupied by the MAP-2- or GFAP-positive structures in the field was measured by selecting the "Analyze Particles" command of the Scion Image program. In this way, the area of individual particles (i.e., MAP-2- or GFAP-immunoreactive structures) in the selected field was measured, and the sum area of all particles was stored for further calculations and statistical analysis. Since spatial calibration of the image was performed in micrometers using the "Set Scale" function of the program prior to measurements, the results from area measurements were obtained in square micrometers and converted later into square millimeters.

RESULTS

General Observations

The clinical condition of animals treated with daily dermal application of DEET, permethrin, or the combination of DEET and permethrin was not different from that of control animals. In addition, statistically, no differences were observed in the weights of animals between the control and treated groups (Fig. 1).

Histopathological Changes

Evaluation of brain sections stained with hematoxylin and eosin (H&E) clearly revealed neuronal degeneration in rats treated with DEET, permethrin, or the combination of DEET and permethrin, in comparison to vehicle-treated rats. Degenerating neurons were characterized by eosinophilic staining of both cell body and proximal dendrites. In contrast, the healthy neurons in the same section exhibited hematoxylin-stained nuclei (with clear nucleoli) and eosin-stained perinuclear cytoplasm. The brain regions where neuronal degeneration was obvious include the motor cerebral cortex, the dentate gyrus, the CA1 and CA3 subfields of the hippocampus, and the Purkinje cell layer of the cerebellum. Other areas of the brain, though, showed occasional dying (eosinophilic) neurons in some animals; the overall cytoarchitecture remained comparable to that of control (vehicle-treated) rats. Therefore, detailed investigation of neuropathological alterations using quantitative methods was performed only on the above brain regions.

Alterations in the Cytoarchitecture of the Motor Cerebral Cortex

In animals treated with either DEET or permethrin alone, both superficial and deeper regions of the motor cortex exhibited degenerating neurons in H&E-stained sections. In superficial regions (layers I–III, Fig. 2), degenerating neurons were conspicuous in both layers II and III. The majority of degenerating neurons in these layers were of the pyramidal type with prominent eosinophilic apical dendrites (Fig. 2 (A₂, A₃)). The overall degree of neuronal degeneration was comparable in animals treated with DEET and animals treated with permethrin. In deeper regions of the cortex (layers IV–VI), degenerating neurons were observed mostly in layer V. These are larger pyramidal neurons with prominent apical and basal dendrites emanating from a larger pyramid-shaped cell body (Fig. 3 (A₂, A₃)). The extent of degeneration appeared greater with exposure to DEET than with exposure to permethrin. Further, in addition to the presence of many degenerating neurons, both superficial and deeper regions of the cortex in animals treated with either DEET or permethrin exhibited clearly reduced packing density of surviving neurons, in comparison to the cortex of control animals (Figs. 2 (A₁–A₃) and 3 (A₁–A₃)). The adjacent sections stained for MAP-2 revealed significantly reduced MAP-2 positive dendrites in layers III and V of animals treated with either DEET or permethrin, in comparison to control animals (Figs. 2 (B₁–B₃) and 3 (B₁–B₃)). MAP-2 expression in dendrites also appeared somewhat disrupted and scarcer. Further, immunostaining with GFAP demonstrated hypertrophy of astrocytes with increased GFAP expression in animals treated with either DEET or permethrin compared with control animals (Figs. 2 (C₁–C₃) and 3 (C₁–C₃)). Thus, both

MAP-2- and GFAP-immunostained sections clearly corroborated the DEET- and permethrin-induced neurodegeneration, as observed in H&E-stained samples.

In animals treated with both DEET and permethrin, degenerating (or eosinophilic) neurons were infrequent in both superficial and deeper regions of the cortex (Figs. 2 (A₄) and 3 (A₄)). The packing density of surviving neurons, however, appeared less than that of control animals (Figs. 2 (A₁, A₄) and 3 (A₁, A₄)). Areas devoid of neurons were conspicuous in layers III and V of the cortex (Figs. 2 (A₄) and 3 (A₄)). Thus, the lack of degenerating (eosinophilic) neurons in animals receiving both DEET and permethrin appeared to be due to early cell death of neurons following the combined exposure in comparison to animals receiving either DEET or permethrin alone. The adjacent sections stained for MAP-2 substantiated the above finding by exhibiting reduced MAP-2 staining of dendrites, particularly in layer V of the cortex (Figs. 2 (B₄, C₄) and 3 (B₄, C₄)). The GFAP-positive astrocytes were of reactive type and exhibited characteristic GFAP expression in their soma. In addition, the pattern of MAP-2 expression differed from those of both control animals and animals treated with either DEET or permethrin by showing a lack of expression in soma of neurons, and by their wavy and fragmented appearance in dendrites throughout the thickness of the cortex (Figs. 2 (B₄) and 3 (B₄)).

Extent of Neuron Loss, Reductions in MAP-2 Immunoreactivity, and Upregulation of GFAP Immunoreactivity in the Motor Cortex

Quantification of healthy (or surviving) neurons per square millimeter of layers III and V of the motor cortex revealed that animals treated with DEET, permethrin, or a combination of DEET and permethrin exhibited a significant decrease in the number of surviving neurons in both layers III and V ($P < 0.01$; Fig. 4), in comparison to control animals. Further comparison between treated groups revealed that the extent of reductions in the density of healthy neurons within the motor cortex was similar in the three treatment groups. Analysis of dying neurons revealed that, in layer III of the motor cortex, animals treated with DEET exhibited a significant number of dying neurons, in comparison to control animals ($P < 0.01$) (Fig. 4). With respect to layer V of the motor cortex, animals treated with either DEET or permethrin exhibited a significant number of dying neurons compared with control animals ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 4). Thus, subchronic dermal application of DEET and permethrin, alone or in combination, leads to a significant reduction in the number of surviving neurons in the motor cortex, and the extent of overall reductions in neurons is similar in the three treatment groups. This suggests that concurrent application of DEET and permethrin does not induce enhanced neu-

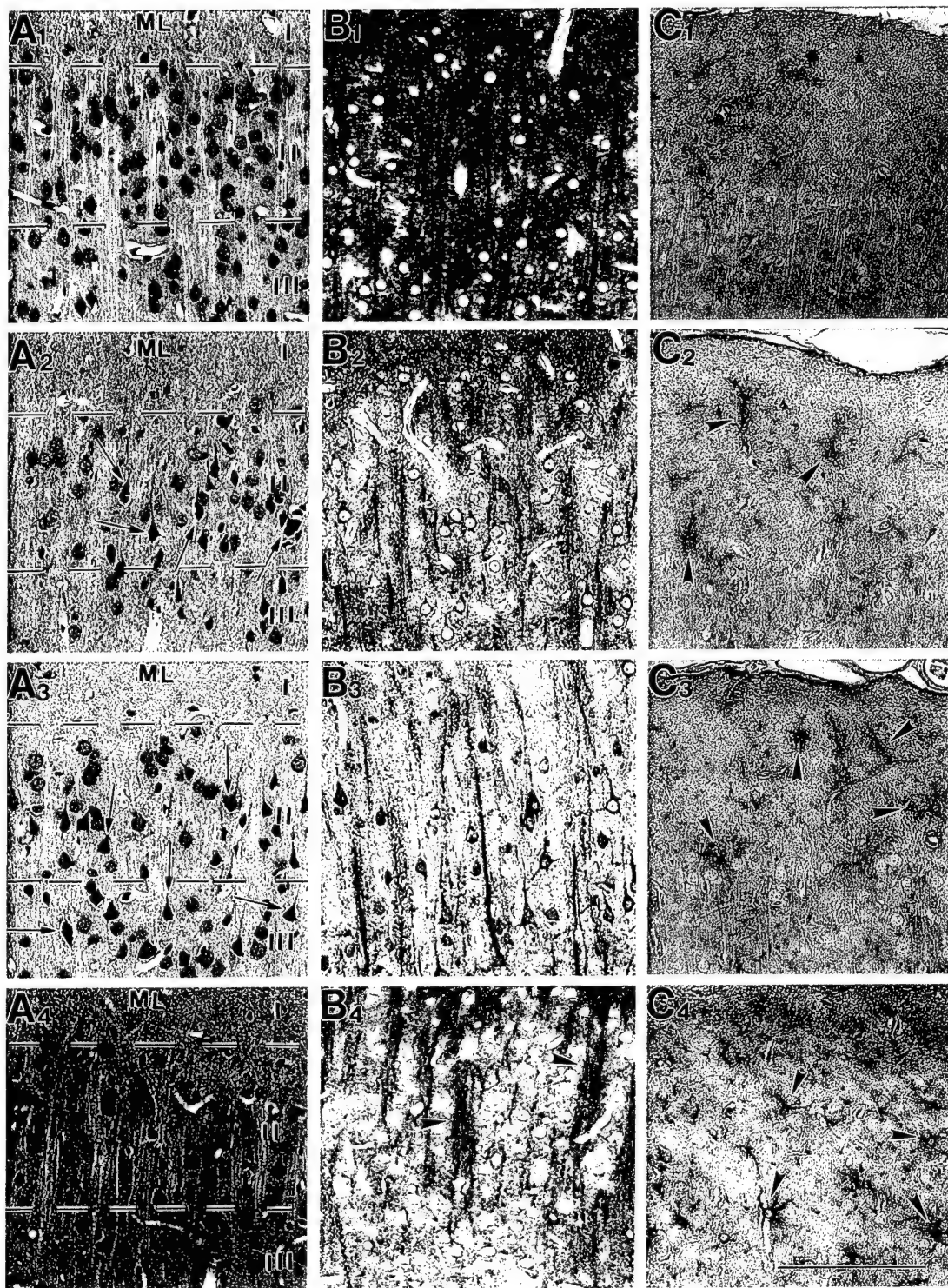


FIG. 2. Alterations in the superficial layers (layers I–III) of the motor cortex following daily dermal application of DEET and permethrin. (A₁–A₄) H&E staining, (B₁–B₄) MAP-2 immunostaining, (C₁–C₄) GFAP immunostaining. (A₁, B₁, C₁) Examples from a control rat. (A₂, B₂, C₂) Examples from a rat treated with DEET. (A₃, B₃, C₃) Examples from a rat treated with permethrin. (A₄, B₄, C₄) Examples from a rat treated with both DEET and permethrin. A large number of degenerating neurons are clearly visible in rats treated with either DEET or permethrin (arrows in A₂ and A₃). Whereas in the rat treated with both DEET and permethrin, vacant areas devoid of neurons (A₄) and wavy appearance of dendrites (arrows in B₄) are conspicuous. Note that, in all three treated groups, the overall MAP-2 immunoreactivity is significantly reduced (B₂–B₄) and GFAP immunoreactivity of astrocytes is significantly enhanced (arrowheads in C₂–C₄), in comparison to the control group (B₁, C₁). Bar = 100 μ m.

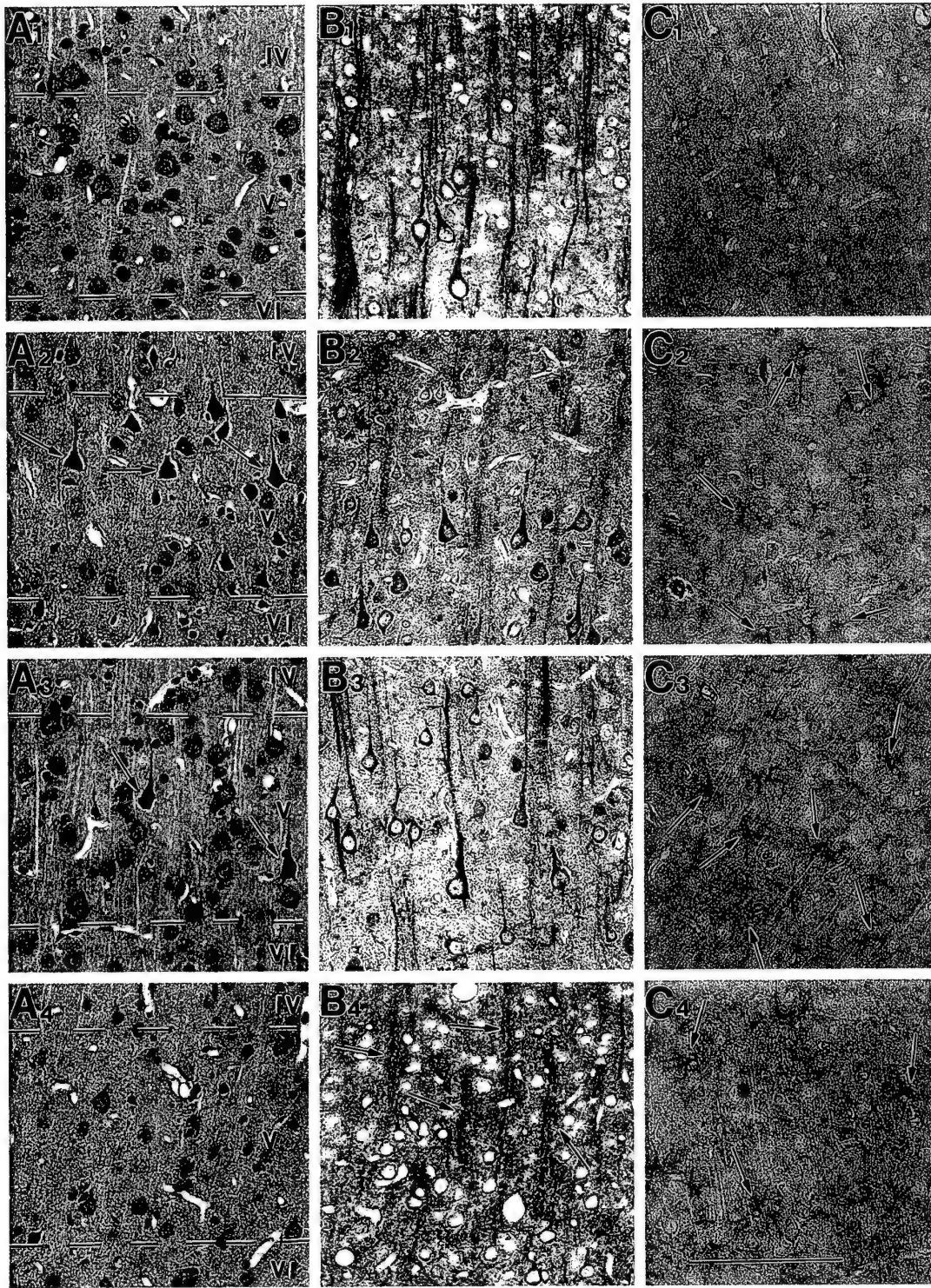


FIG. 3. Changes in the deeper layers (layers IV and V) of the motor cortex following daily application of DEET and permethrin. (A₁–A₄) H&E staining, (B₁–B₄) MAP-2 immunostaining, (C₁–C₄) GFAP immunostaining. (A₁, B₁, C₁) Examples from a control rat. (A₂, B₂, C₂) Examples from a rat treated with DEET. (A₃, B₃, C₃) Examples from a rat treated with permethrin. (A₄, B₄, C₄) Examples from a rat treated with both DEET and permethrin. A large number of degenerating pyramidal neurons are clearly visible in layer V of the cortex in rats treated with either DEET or permethrin (arrows in A₂ and A₃). In the rat treated with both DEET and permethrin, vacant areas devoid of neurons (A₄) and wavy appearance of dendrites (arrows in B₄) are conspicuous. Note that, in all three treated groups, the overall MAP-2 immunoreactivity is significantly reduced (B₂–B₄) and GFAP immunoreactivity of astrocytes is significantly enhanced (arrows in C₂–C₄), in comparison to the control group (B₁, C₁). Bar = 100 μ m.

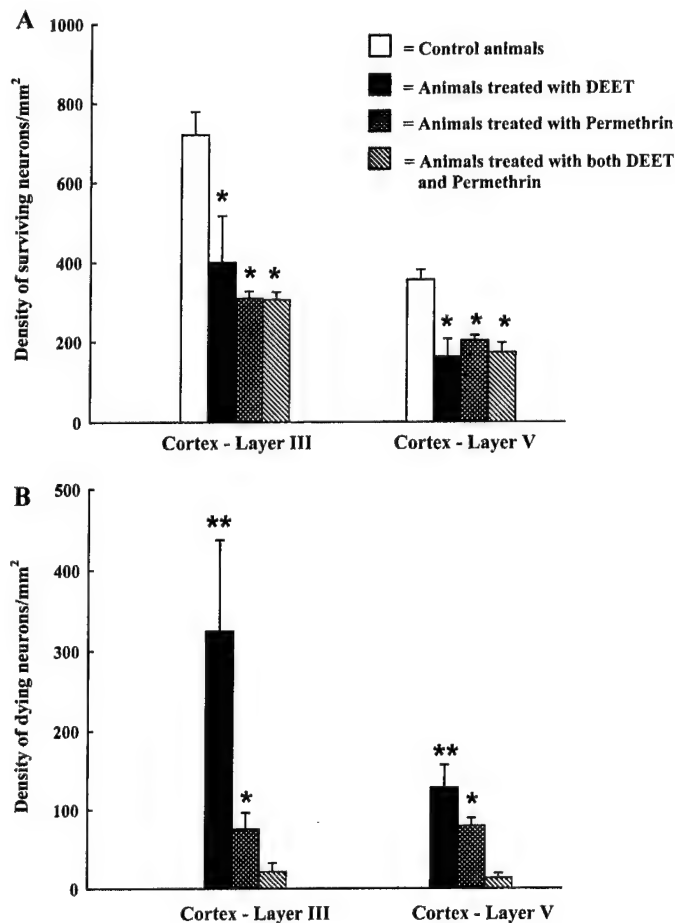


FIG. 4. Histograms show the density of surviving (A) and dying (B) neurons per square millimeter of layers III and V of the motor cortex. Values represent means and standard errors ($n = 5$ per group). Analyses with one-way ANOVA show significant differences for surviving neurons between groups ($P < 0.01$ in layer III, $P < 0.001$ in layer V). The post hoc analysis with the Student Newman-Keuls multiple comparison test further revealed that animals treated with DEET or permethrin alone exhibit a significant decrease in the number of surviving neurons, in comparison to control animals (layer III, $P < 0.05$; layer V, $P < 0.01$). Analysis of dying neurons revealed that, in layer III of the motor cortex, animals treated with DEET exhibit a significant increase in the number of dying neurons, in comparison to control animals, animals treated with permethrin, and animals treated with both DEET and permethrin ($P < 0.01$). In layer V of the motor cortex, animals treated with either DEET or permethrin exhibit a significant increase in dying neurons compared with control animals ($P < 0.001$ and $P < 0.01$, respectively). Further, exposure to DEET alone results in a significantly increased number of dying neurons than exposure to permethrin alone ($P < 0.05$) and exposure to both DEET and permethrin ($P < 0.001$).

ron loss in the motor cortex, compared with exposure to either DEET or permethrin.

Quantification of the area of MAP-2-immunoreactive elements per unit area of layers III and V of the motor cortex showed that there were fewer MAP-2-positive structures in the motor cortex of all treated groups (Fig. 5A). In layer III of the cortex, the MAP-2-immunoreactive structures exhibited 27–28% reduction with

exposure to DEET or permethrin alone ($P < 0.05$) and 15% reduction with exposure to both DEET and permethrin ($P > 0.05$), whereas in layer V of the motor cortex, the MAP-2-immunopositive structures showed 52% reduction with exposure to DEET alone ($P < 0.01$), 35% reduction with exposure to permethrin alone ($P < 0.05$), and 49% reduction with exposure to both DEET and permethrin ($P < 0.01$). The measurement of GFAP-immunoreactive structures per unit area of layer V of the motor cortex revealed a significant upregulation in GFAP-positive elements ($P < 0.05$) with exposure to either DEET (74% increase) or permethrin (63% increase) (Fig. 5B). However, with

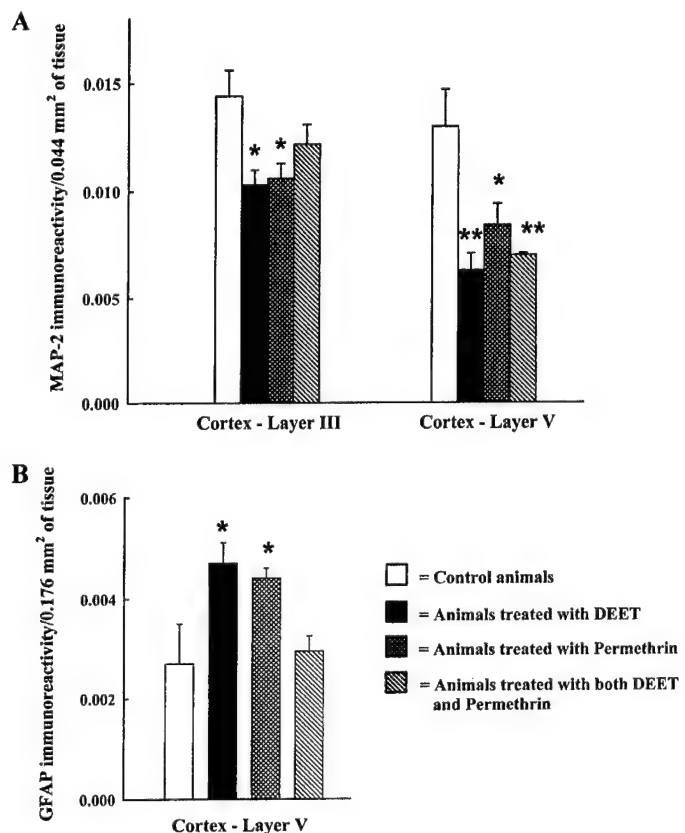


FIG. 5. Histograms in (A) show the area of MAP-2-immunoreactive elements in square millimeter per unit area (0.044 mm^2) of layers III and V of the motor cortex. Values represent means and standard errors ($n = 5$ per group). In layer III of the cortex, the MAP-2-immunoreactive structures exhibited 27–28% reduction with exposure to DEET or permethrin alone ($P < 0.05$), and 15% reduction with exposure to both DEET and permethrin ($P > 0.05$). Whereas in layer V of the motor cortex, the MAP-2-immunopositive structures showed 52% reduction with exposure to DEET alone ($P < 0.01$), 35% reduction with exposure to permethrin alone ($P < 0.05$), and 49% reduction with exposure to both DEET and permethrin ($P < 0.01$). Histograms in (B) show the area of GFAP-immunoreactive elements (in mm^2) per unit area (0.176 mm^2) of layer V of the motor cortex. Values represent means and standard errors ($n = 5$ per group). Note a significant upregulation in GFAP-positive elements ($P < 0.05$) with exposure to either DEET (74% increase) or permethrin (63% increase). However, with combined exposure to DEET and permethrin, there was only a 10% increase in GFAP immunoreactivity ($P > 0.05$).

combined exposure to DEET and permethrin, there was only a 10% increase in GFAP immunoreactivity ($P > 0.05$).

Alterations in the Hippocampal Formation

Neuronal degeneration was obvious in the dentate gyrus and CA1 and CA3 subfields of the hippocampal formation following exposure to DEET and permethrin, alone or in combination. In all treated groups, the thickness and cell packing density of the granule cell layer appeared reduced compared with that of control animals. Further, in dentate gyrus of animals treated with DEET and permethrin, degenerating neurons were observed in both the granule cell layer and the dentate hilus (Fig. 6 (A_2 , A_3)). GFAP immunoreactivity was enhanced in the molecular layer and the hilus of all three treated groups, in comparison to control animals (Fig. 6 (B_1 – B_4)). MAP-2 staining of the granule cell layer and the molecular layer did not show any significant differences between animals belonging to different treated groups but appeared slightly reduced compared with that of the control animals (data not shown).

In the CA1 subfield of the hippocampus, the thickness and cell packing density of stratum pyramidale appeared reduced in treated groups compared with control animals (Fig. 7). Further, degenerating neurons were clearly observed in the stratum pyramidale of animals treated with either DEET or permethrin alone (Fig. 7 (A_1 – A_3)). MAP-2 staining of adjacent sections demonstrated a conspicuously reduced density of MAP-2-positive dendrites in animals belonging to all three treated groups compared with control animals (Fig. 7 (B_1 – B_4)). In animals treated with DEET, MAP-2-positive apical dendrites in stratum radiatum were thinner and appeared disrupted, whereas in animals treated with permethrin, MAP-2-positive dendrites appeared to be either beaded or arranged in aggregates, with highly conspicuous vacant spaces between them. In animals treated with both DEET and permethrin, MAP-2-positive dendrites were wavy and thinner. The appearance of MAP-2 staining of apical dendrites in all treated groups highly contrasted with the homogenous MAP-2 staining observed in control animals. Immunostaining of neighboring sections for GFAP demonstrated enhanced GFAP immunoreactivity in animals belonging to all three treated groups compared with control animals (Fig. 7 (C_1 – C_4)).

In the CA3 subfield of the hippocampus, the thickness and neuronal density of stratum pyramidale appeared reduced in all three treated groups compared with control animals (Fig. 8). The degenerating neurons were conspicuous in the stratum pyramidale of animals treated with DEET alone (Fig. 8 (A_1 , A_2)). MAP-2 staining of adjacent sections demonstrated only a slightly reduced density of MAP-2-positive dendrites

in animals belonging to all three treated groups compared with control animals (data not shown). GFAP immunostaining of neighboring sections showed enhanced GFAP immunoreactivity in animals belonging to all three treated groups compared with control animals (Fig. 8 (C_1 – C_4)).

Extent of Neuron Loss, Reductions in MAP-2

Immunoreactivity, and Upregulation of GFAP

Immunoreactivity in the Hippocampal Formation

Quantification of surviving and dying neurons per square millimeter of granule cell layer of the dentate gyrus and pyramidal cell layer of subfields CA1 and CA3 (Fig. 9) demonstrated the following. In dentate granule cell layer, animals treated with DEET, permethrin, or a combination of DEET and permethrin exhibited a significant decrease in surviving neurons, in comparison to control animals ($P < 0.01$) (Fig. 9). Further, animals treated with either DEET or permethrin exhibited a significant decrease in surviving neurons, in comparison to animals treated with combined DEET and permethrin ($P < 0.01$) (Fig. 9). Analysis of dying neurons showed that animals treated with either DEET or permethrin exhibited a significant number of dying neurons ($P < 0.05$) (Fig. 9). In the CA1 subfield, only animals treated with either DEET or permethrin exhibited a significant decrease in the number of surviving neurons compared with control animals ($P < 0.05$) (Fig. 9). Analysis of dying neurons also showed the same trend. In the CA3 subfield, animals treated with DEET, permethrin, or a combination of DEET and permethrin exhibited a significant decrease in surviving neurons, in comparison to control animals ($P < 0.01$) (Fig. 9). Analysis of dying neurons revealed that animals treated with either DEET or permethrin exhibited a significant number of dying neurons ($P < 0.01$). Thus, a significant reduction in surviving neurons occurs in the dentate gyrus and CA3 subfield of the hippocampal formation following subchronic dermal application of DEET and permethrin, alone or in combination; however, the overall neuron loss in the dentate gyrus is significantly greater when DEET and permethrin are applied separately. In the CA1 subfield of the hippocampus, a significant decrease in the number of surviving neurons occurs with exposure to DEET or permethrin alone but not with exposure to both DEET and permethrin.

Quantification of the area of MAP-2-immunoreactive elements per unit area of the CA1 stratum radiatum showed that there were fewer MAP-2-positive structures in the CA1 subfield of all treated groups (Fig. 10A). The MAP-2-immunoreactive structures exhibited 28% reduction with exposure to DEET alone ($P < 0.05$), and 16% reduction with exposure to permethrin alone or exposure to both DEET and permethrin ($P < 0.05$). The measurement of GFAP-immunoreactive

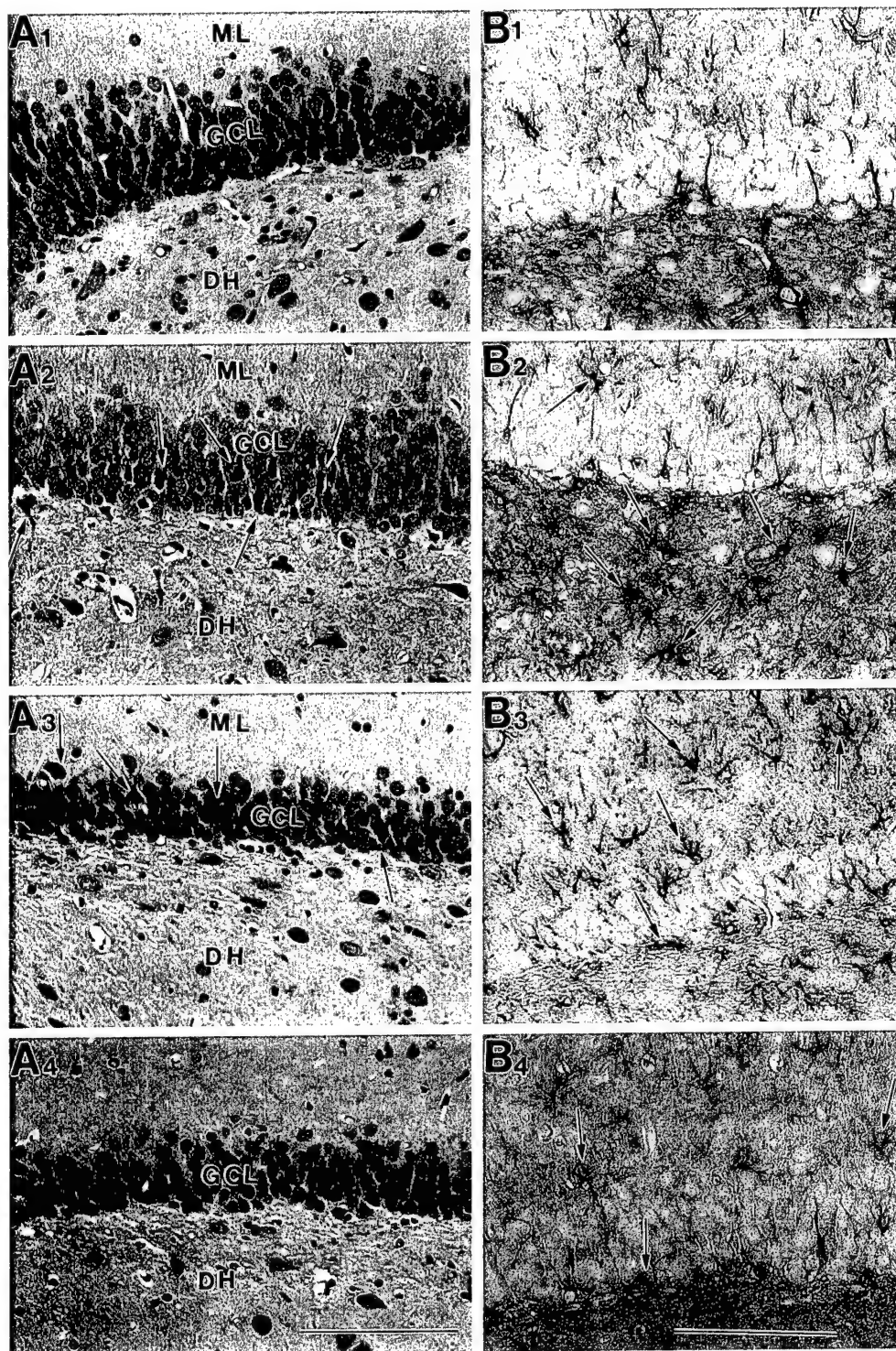


FIG. 6. Alterations in the dentate gyrus following daily application of DEET and permethrin. (A₁–A₄) H&E staining, (B₁–B₄) GFAP immunostaining. (A₁, B₁) Examples from a control rat. (A₂, B₂) Examples from a rat treated with DEET. (A₃, B₃) Examples from a rat treated with permethrin. (A₄, B₄) Examples from a rat treated with both DEET and permethrin. A large number of degenerating neurons are clearly visible in the dentate granule cell layer (GCL) and the dentate hilus (DH) of rats treated with either DEET or permethrin (arrows in A₂ and A₃). In the rat treated with both DEET and permethrin (A₄), both thickness and cell packing density of granule cell layer are reduced compared with the control rat. Note that GFAP immunoreactivity is upregulated in all three treated groups (B₂, B₃, B₄). ML, molecular layer. Bar = 100 μ m.

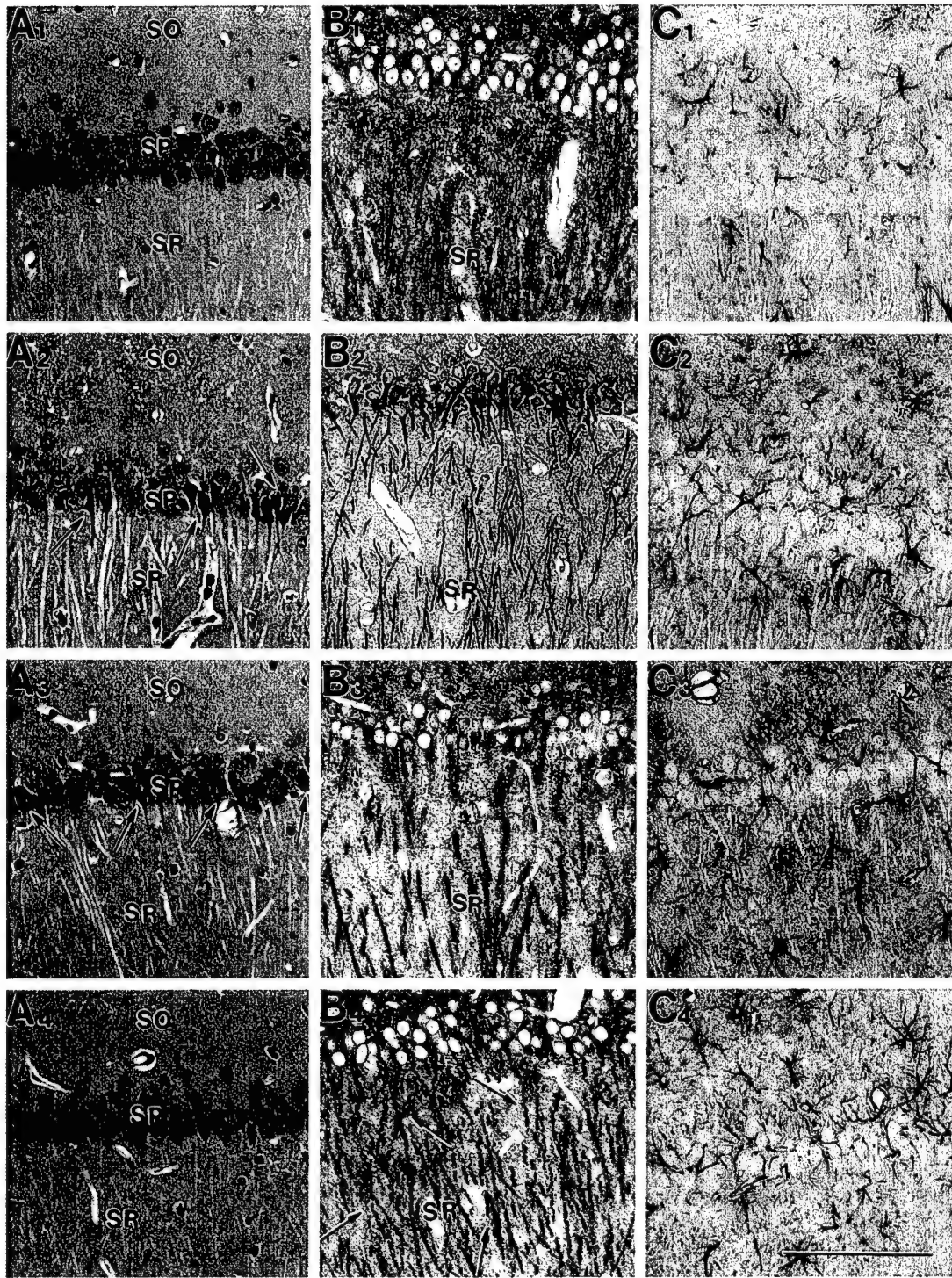


FIG. 7. Alterations in the CA1 subfield of the hippocampus following daily dermal application of DEET and permethrin. (A₁–A₄) H&E staining, (B₁–B₄) MAP-2 immunostaining, (C₁–C₄) GFAP immunostaining. (A₁, B₁, C₁) Examples from a control rat. (A₂, B₂, C₂) Examples from a rat treated with DEET. (A₃, B₃, C₃) Examples from a rat treated with permethrin. (A₄, B₄, C₄) Examples from a rat treated with both DEET and permethrin. A large number of degenerating pyramidal neurons are clearly visible in the stratum pyramidale (SP) of rats treated with either DEET or permethrin (arrows in A₂ and A₃). In the rat treated with both DEET and permethrin, both thickness and cell packing density of CA1 cell layers are reduced compared with the control rat (A₄), and dendrites in stratum radiatum have a wavy appearance (B₄). Note that, in all three treated groups, the overall density of MAP-2-immunoreactive elements is significantly reduced (B₂–B₄) and the pattern of MAP-2 expression in dendrites is altered, in comparison to the control group (B₁). Further, in all treatment groups (C₂–C₄), GFAP-immunoreactive astrocytes are significantly increased in both stratum oriens (SO) and stratum radiatum (SR). Bar = 100 μ m.

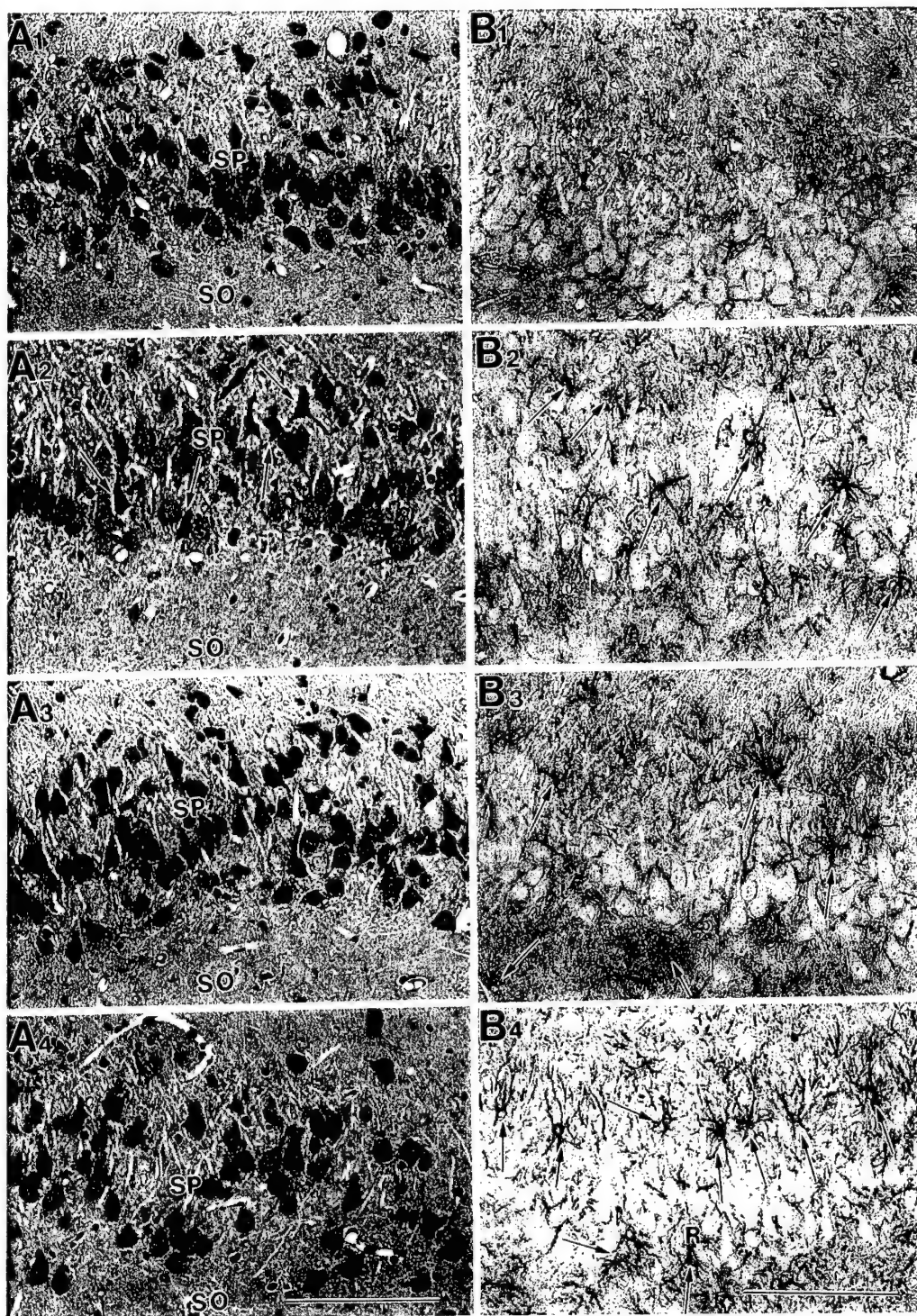


FIG. 8. Changes in the CA3 subfield of the hippocampus following daily application of DEET and permethrin. (A₁–A₄) H&E staining, (B₁–B₄) GFAP immunostaining. (A₁, B₁) Examples from a control rat. (A₂, B₂) Examples from a rat treated with DEET. (A₃, B₃) Examples from a rat treated with permethrin. (A₄, B₄) Examples from a rat treated with both DEET and permethrin. A large number of degenerating neurons are clearly visible in the stratum pyramidale (SP) of the CA3 subfield of the rat treated with DEET alone (arrows in A₂). In rats treated with permethrin alone (A₃) and both DEET and permethrin (A₄), the thickness and cell packing density of the CA3 cell layer are reduced compared with the control rat. Note that GFAP immunoreactivity is upregulated in all three treated groups (B₂, B₃, B₄). SO, stratum oriens. Bar = 100 μ m.

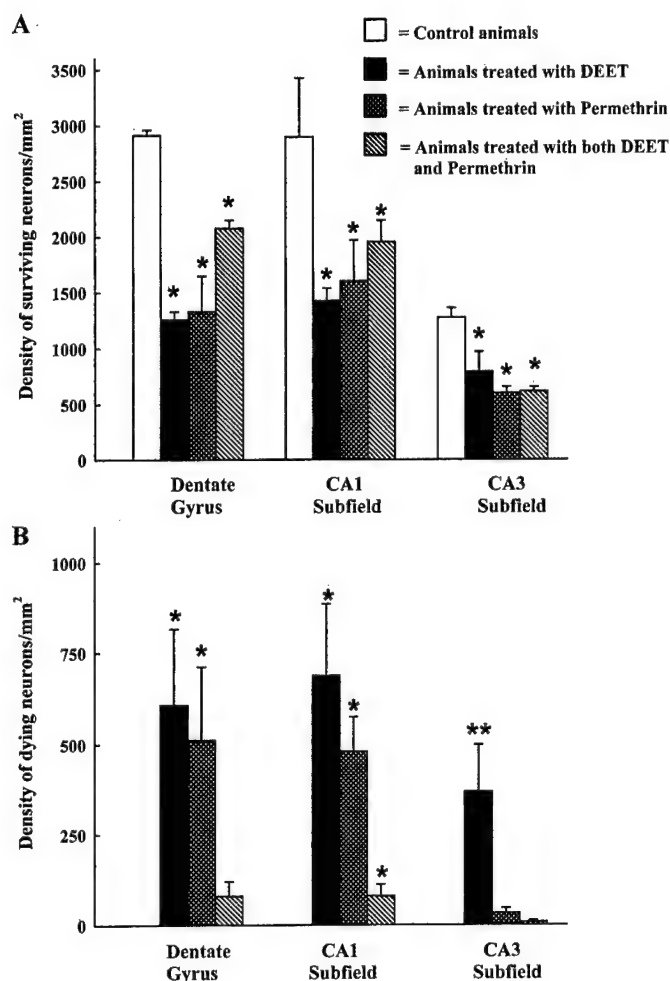


FIG. 9. Histograms show the density of surviving (A) and dying (B) neurons per square millimeter of area of different cell layers of the hippocampal formation. Values represent means and standard errors ($n = 5$ per group). Analyses with one-way ANOVA revealed significant differences between groups for both surviving neurons (dentate gyrus, $P < 0.001$; CA1 subfield, $P < 0.05$; CA3 subfield, $P < 0.001$) and dying neurons (dentate gyrus, CA1 and CA3 subfields, $P < 0.01$). The post hoc analysis with Student Newman-Keuls multiple comparisons test further revealed the following. In dentate granule cell layer, animals treated with DEET, permethrin, or a combination of DEET and permethrin exhibit a significant decrease in surviving neurons, in comparison to control animals ($P < 0.01$). Further, animals treated with either DEET or permethrin exhibit a significant decrease in surviving neurons, in comparison to animals treated with both DEET and permethrin ($P < 0.01$). Analysis of dying neurons (B) shows that animals treated with either DEET or permethrin exhibit a significant number of dying neurons compared with control animals ($P < 0.05$); the number of dying neurons with exposure to DEET alone is greater than with exposure to both DEET and permethrin ($P < 0.05$). In CA1 subfield, only animals treated with either DEET or permethrin exhibit a significant decrease in the number of surviving neurons compared with controls ($P < 0.05$). Analysis of dying neurons shows that animals treated with either DEET or permethrin exhibit a greater number of dying neurons than both controls ($P < 0.01$) and animals treated with both DEET and permethrin ($P < 0.05$). In CA3 subfield, animals treated with DEET, permethrin, or a combination of DEET and permethrin exhibit a significant decrease in surviving neurons, in comparison to controls ($P < 0.01$). Analysis of dying neurons reveals that animals treated with either DEET or permethrin exhibit a greater number of dying neurons compared with both controls ($P < 0.01$) and animals treated with both DEET and permethrin ($P < 0.05$).

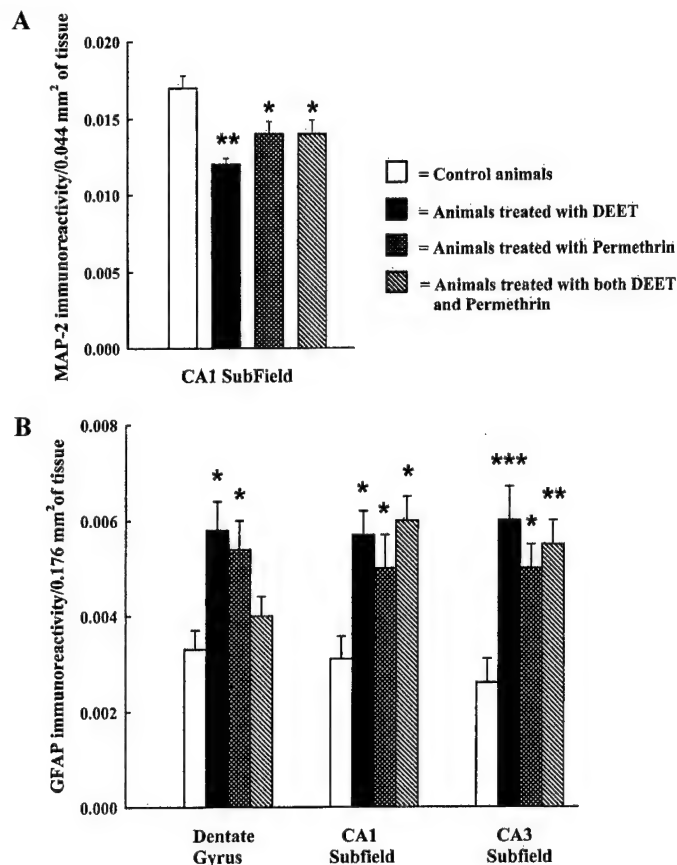


FIG. 10. Histograms in (A) show the area of MAP-2-immunoreactive elements (in mm²) per unit area (0.044 mm²) of CA1 stratum radiatum of the hippocampus. Values represent means and standard errors ($n = 5$ per group). Note that there are fewer MAP-2-positive structures in the CA1 subfield of all treated groups. The MAP-2-immunoreactive structures exhibited 28% reduction with exposure to DEET alone ($P < 0.05$), and 16% reduction with exposure to permethrin alone or exposure to both DEET and permethrin ($P < 0.05$). Histograms in (B) show the area of GFAP-immunoreactive elements (in mm²) per unit area (0.176 mm²) of different regions of the hippocampus. Values represent means and standard errors ($n = 5$ per group). Note the upregulation in GFAP-positive elements within all regions of the hippocampus in all treatment groups. In dentate gyrus, the increase was 77% with DEET exposure ($P < 0.05$), 65% with permethrin exposure ($P < 0.05$), and 24% with exposure to both DEET and permethrin ($P < 0.05$). In the CA1 subfield, the increase in different treatment groups varied from 81 to 91% ($P < 0.05$). In the CA3 subfield, the increase in GFAP immunoreactivity varied from 60 to 93% ($P < 0.05$).

structures per unit area of different regions of the hippocampal formation demonstrated upregulation in GFAP-positive elements in all treatment groups (Fig. 10B). In dentate gyrus, the increase was 77% with DEET exposure ($P < 0.05$), 65% with permethrin exposure ($P < 0.05$), and 24% with exposure to both DEET and permethrin ($P < 0.05$). In the CA1 subfield, the increase in different treatment groups varied from 81 to 91% ($P < 0.05$). In the CA3 subfield, the increase in GFAP immunoreactivity varied from 60 to 93% ($P < 0.05$).

Alterations in the Cytoarchitecture of the Cerebellum

In the cerebellum, the most conspicuous damage following exposure to DEET and permethrin, alone or in combination, was in the Purkinje cell layer. A large number of degenerating neurons were observed in animals treated with either DEET or permethrin compared with control animals (Fig. 11 (A₁–A₃)). In animals treated with combined DEET and permethrin, dying neurons were infrequent. However, the Purkinje cell density per length of Purkinje cell layer appeared reduced in comparison to control animals (Fig. 11 (A₄)). Wide areas of the Purkinje cell layer lacking Purkinje neurons were frequently encountered in animals treated with both DEET and permethrin (Fig. 11 (A₄)). Both thickness and cell packing density in the granule cell layer appeared comparable in control animals and animals belonging to three treated groups. GFAP immunostaining of neighboring sections showed significantly enhanced GFAP immunoreactivity in the cerebellar white matter of animals belonging to the three treatment groups compared with control animals (Fig. 11 (B₁–B₄)). However, the maximal enhancement of GFAP immunoreactivity was observed in animals treated with both DEET and permethrin (Fig. 11 (B₄)).

Extent of Purkinje Neuron Loss and Upregulation of GFAP Immunoreactivity in the Cerebellum

Quantitative analysis of Purkinje cells showed that animals treated with DEET and permethrin, alone or in combination, exhibited a significant decrease in surviving neurons, in comparison to control animals (76–83% in lobule 2 of the cerebellar vermis, and 36–58% in crus 2 ansiform lobule of the cerebellar hemisphere, $P < 0.001$) (Fig. 12). However, the degree of neuron loss in the cerebellar hemisphere with combined exposure to DEET and permethrin was greater than that with exposure to DEET or permethrin alone ($P < 0.05$) (Fig. 12). Analysis of dying neurons in the cerebellar vermis revealed that animals treated with either DEET or permethrin exhibited a significant number of dying neurons ($P < 0.001$) (Fig. 12). However, in the cerebellar hemisphere, all treated groups exhibited a significant number of dying neurons ($P < 0.05$). Thus, as in the cerebral cortex and the hippocampal formation, significant neuronal cell death occurs in the Purkinje cell layer of the cerebellum following subchronic dermal application of DEET and permethrin, alone or in combination; however, the overall neuron loss in the cerebellar hemisphere is significantly greater with combined application of DEET and permethrin, compared with exposure to DEET or permethrin alone. The measurement of GFAP-immunoreactive structures per unit area of the central white matter of the cerebellum demonstrated significant upregulation in GFAP-positive elements in all treatment groups: 53–60% increase with exposure to DEET or permethrin ($P < 0.05$) (Fig.

12C), and 106% increase with exposure to both DEET and permethrin ($P < 0.01$) (Fig. 12C).

DISCUSSION

The present study was designed to investigate the effects of daily dermal application of DEET and permethrin, alone or in combination, for 60 days on histopathological changes in the brain of male rats. The route of exposure and dose levels of test compounds were chosen to closely reflect those present during the Persian Gulf War (1). The test compounds were applied dermally at a dose that was approximately equivalent to the exposure that may have occurred to army personnel during the Gulf War (18; W. McCain, Department of Defense, personal communication). Our data suggest that exposure to DEET and permethrin, alone or in combination, for 60 days causes the following: (1) a diffuse neuronal cell death in the motor cortex, the different subfields of the hippocampal formation, and the Purkinje cell layer of the cerebellum; (2) a significant reduction in MAP-2-positive immunoreactive structures associated with atypical expression of MAP-2 in dendrites of surviving neurons within the cerebral cortex and the hippocampus (the expression of MAP-2 within apical dendrites of pyramidal neurons of the cortex and the CA1 subfield was characterized by a beaded, disrupted, or wavy appearance); (3) a significant upregulation of GFAP-positive structures (this was exemplified by GFAP expression in soma of astrocytes and hypertrophy of astrocytic processes emanating from the soma).

To determine the overall extent of neuron loss, we quantified the density of surviving (healthy) and dying neurons in layers III and V of the motor cerebral cortex, granule cells of the dentate gyrus, pyramidal neurons of hippocampal CA1 and CA3 subfields, and Purkinje cells of the cerebellum. Neuronal cell death was evident in all three treated groups by a significant decrease in the density of surviving neurons. In animals treated with DEET or permethrin alone, the occurrence of neuronal cell death was also confirmed by the presence of a significant number of dying (eosinophilic) neurons after the 60-day exposure regimen. However, in animals treated with both DEET and permethrin, the number of dying neurons after the same exposure regimen was significantly less than in animals treated with DEET or permethrin alone. A lack of significant number of dying neurons but a clear reduction in the number of surviving neurons (in comparison to control animals) in animals exposed to both DEET and permethrin suggests that, in animals receiving combined DEET and permethrin, neuronal cell death occurs earlier than in animals receiving either DEET or permethrin alone. However, analysis of dying neurons at multiple time points during the exposure period employed in this study is necessary to clearly address the above issue. Further, the extent of reductions in

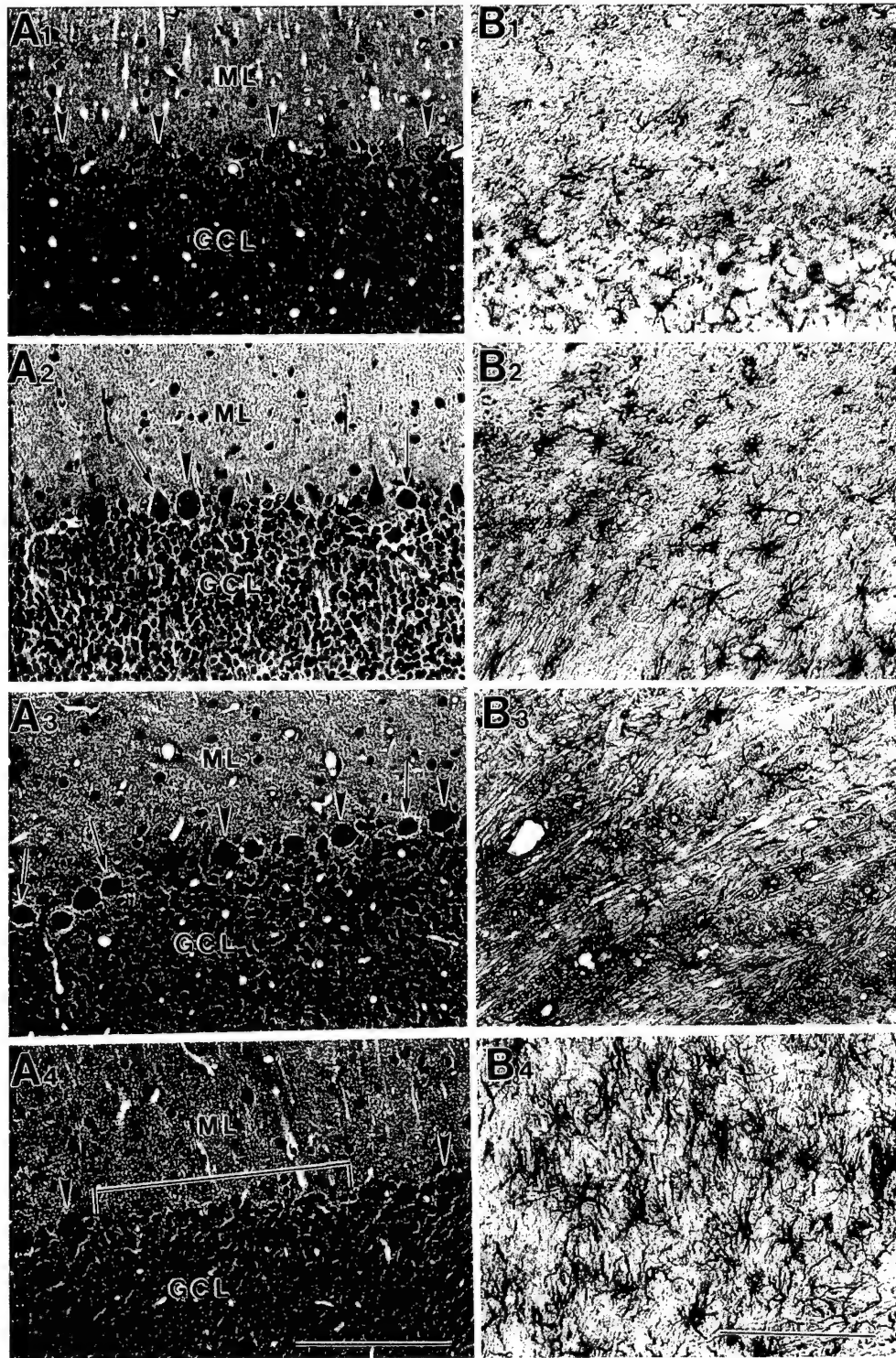


FIG. 11. Alterations in the cerebellum following daily application of DEET and permethrin. (A₁–A₄) H&E staining of the cerebellar cortex, (B₁–B₄) GFAP immunostaining of the cerebellar white matter. (A₁, B₁) Examples from a control rat. (A₂, B₂) Examples from a rat treated with DEET. (A₃, B₃) Examples from a rat treated with permethrin. (A₄, B₄) Examples from a rat treated with both DEET and permethrin. A large number of degenerating Purkinje neurons are clearly visible in the Purkinje cell layer of rats treated with either DEET or permethrin (arrows in A₂, A₃). Arrows in (A₁)–(A₄) point to surviving neurons. In rats treated with both DEET and permethrin (A₄), a large area of Purkinje cell layer is devoid of Purkinje neurons (bracketed area, A₄). Note that GFAP immunoreactivity in the white matter of the cerebellum is significantly upregulated in all three treated groups (B₂, B₃, B₄), with maximal upregulation in the group treated with both DEET and permethrin. GCL, granule cell layer; ML, molecular layer. Bar = 100 μ m.

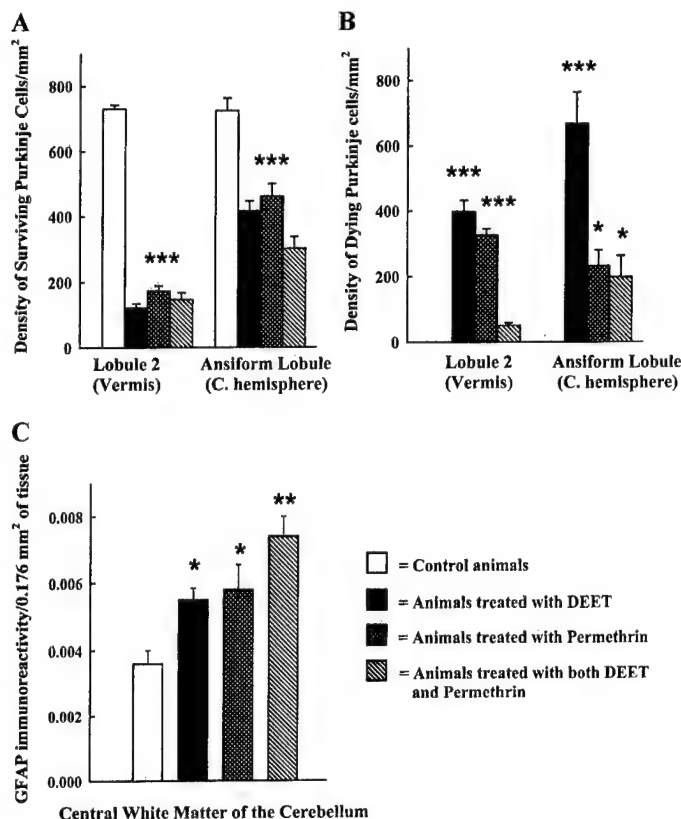


FIG. 12. Histograms show the density of surviving (A) and dying (B) neurons per square millimeter of area of the Purkinje cell layer in lobule 2 of the cerebellar vermis and crus 2 ansiform lobule of the cerebellar hemisphere. Values represent means and standard errors ($n = 5$ per group). Note that animals treated with DEET and permethrin, alone or in combination, exhibit a significant decrease in surviving neurons, in comparison to control animals (76–83% in lobule 2 of the cerebellar vermis, and 36–58% in crus 2 ansiform lobule of the cerebellar hemisphere, $P < 0.001$). And, the degree of neuron loss in the cerebellar hemisphere with combined exposure to DEET and permethrin is greater than that with exposure to DEET or permethrin alone ($P < 0.05$). Also, animals treated with either DEET or permethrin exhibit a significant number of dying neurons in the cerebellar vermis ($P < 0.001$; B). In the cerebellar hemisphere, all treated groups exhibit a significant number of dying neurons ($P < 0.05$). Histograms in (C) show GFAP-immunoreactive elements (in mm^2) per unit area (0.176 mm^2) of the central white matter of the cerebellum. Note that a significant upregulation in GFAP-positive elements occurs in all treatment groups (53–60% increase with exposure to DEET or permethrin, $P < 0.05$; 106% increase with exposure to both DEET and permethrin, $P < 0.01$).

surviving neurons within some regions of the brain varied between the three treatment groups. Layers III and V of the motor region of the cerebral cortex exhibited similar levels of reductions (43–57%) following exposure to DEET and permethrin, alone or in combination. The dentate gyrus of the hippocampal formation demonstrated a significantly greater level of reduction with exposure to DEET or permethrin alone, in comparison to combined DEET and permethrin exposure (54% with DEET alone, 54% with permethrin alone, and 29% with combined DEET and permethrin); however, the CA1 and CA3 subfields of the hippocam-

pus exhibited statistically similar level of reductions (CA1, 33–51% reduction; CA3, 38–53% reduction) following exposure to DEET and permethrin, alone or in combination. The Purkinje cells of the cerebellar vermis exhibited similar levels of reductions with all three exposures (76–83% reduction). However, the Purkinje cells of the cerebellar hemisphere demonstrated a significantly greater reduction with combined exposure to DEET and permethrin (58% decrease) than with exposure to DEET or permethrin alone (36–43% decrease). Thus, in the motor cerebral cortex, the hippocampal subfields CA1 and CA3, and the Purkinje cell layer of the cerebellar vermis, exposure to DEET and permethrin, alone or in combination, causes similar levels of neuronal cell death. In contrast, in the dentate gyrus, exposure to DEET alone causes greater neuronal cell death than exposure to both DEET and permethrin. And, in the Purkinje cell layer of the cerebellar hemisphere, exposure to combined DEET and permethrin causes more damage than exposure to DEET or permethrin alone.

A greater level of neuron loss in the dentate gyrus following exposure to DEET alone compared with exposure to a combination of DEET and permethrin suggests that the extent of DEET-induced neuronal loss in some regions of the brain wanes significantly when both DEET and permethrin are applied together. The reduced neuronal loss with exposure to both DEET and permethrin likely reflects a decrease in effective concentration of chemicals at the neurotoxicity target, as concurrent exposure to chemicals can decrease their absorption (18, 24). This may also suggest that there is some protective effect with concurrent exposure to DEET and permethrin. Nevertheless, animals treated with both DEET and permethrin exhibited maximal cytoarchitectural alterations in the expression of MAP-2 within the motor cerebral cortex and the CA1 subfield of the hippocampus though the overall reductions in MAP-2 immunoreactivity were mostly comparable in all treated groups. The upregulation of GFAP immunoreactivity was comparable in the three treatment groups in the CA3 subfield of the hippocampus. In the motor cortex and the dentate gyrus, the upregulation was greater with exposure to DEET or permethrin alone compared with combined exposure, whereas, in the cerebellum, the upregulation was greater with combined exposure than with exposure to DEET or permethrin alone. The above pattern of cytoskeletal alterations suggests that degenerative changes induced by co-exposure to DEET and permethrin are significant and detrimental to the normal functioning of the central nervous system, despite a slight reduction in the extent of overall neuronal cell loss.

In our previous studies in hens using the subcutaneous route of exposure at relatively higher doses, we demonstrated that co-exposure to DEET and permethrin results in an enhanced level of toxicity com-

pared with exposure to each chemical alone (2). Our recent behavioral studies in rats showed that exposure to DEET or permethrin or both DEET and permethrin for 60 days, using the same dose levels used in the current study, leads to significant deficits in sensorimotor functions (1). In addition, our previous study demonstrated subtle changes in the blood-brain barrier following exposure to either DEET or both DEET and permethrin, and suggested that additional approaches such as histopathological evaluations may provide definitive proof of the changes in the CNS occurring as a consequence of DEET treatment alone or a combination with permethrin. Our present data provide clear histopathological evidence that subchronic exposure to DEET and permethrin, alone or in combination, leads to significant neuronal cell death and cytoskeletal abnormalities in surviving neurons that could compromise functions of the brain.

The neurotoxic effects of DEET may be augmented both by its increased localization into the CNS because of its lipophilicity and because of decrease in the transport of otherwise critical molecules. Severe signs of CNS toxicity due to DEET and permethrin are apparent only at high doses; e.g., DEET-induced signs of CNS depression, death, and protracted seizure activity were observed at several dose levels in rats (39). Similar complications have been observed in DEET poisoning in humans (21, 26). Symptoms such as daytime sleepiness and impaired cognitive functions have been shown to result from heavy DEET exposure, whereas gait balance and dexterity were moderately affected (21). Additionally, lethargy has been noted as a prominent feature in severe acute DEET intoxication (33, 35). A relatively recent study found a decrease in motor activity in male and female rats after a single dose of DEET treatment (39). Permethrin-induced behavioral changes have also been documented in animals (16). Permethrin-induced neurotoxic changes are characterized by aggressive sparring, increased sensitivity to external stimuli, and fine tremors that progresses to whole-body tremors and prostration (4, 37, 38). McDaniel and Moser (22) reported a decrease in grip strength and induced head and forelimb shaking. Additionally, decreased operant response rate and a decrease in turning-wheel activity have been observed (6, 14). Studies by Crofton and Reiter (9) have shown a decrease in locomotor activity in rats exposed to permethrin.

The cytoarchitecture of the CNS is maintained by a complex cellular milieu that involves neurons and a variety of cells of glial origin. For the CNS to function properly and to respond to external stimuli, it is absolutely required that proper communication is maintained within these cells. A major determinant of neuronal morphology is the cytoskeleton. Different components of cytoskeleton within the neurons and astrocytes provide forces to maintain the appropriate cellular structure; e.g., neuronal dendrites and axons

are maintained in stable conditions by the force provided by the elements of cytoskeleton (13). Such interactions are essential for proper synapse formation. The components of cytoskeleton are microfilaments, intermediate filaments, and microtubules. An important neuronal component, MAP-2, is enriched in dendrites and cell bodies (36), in which it stabilizes the polymerized tubulin. Abnormal regulation of expression of MAP-2 causes suppression of neurite outgrowth and reduction in the number of neurites in cultured neurons (7). Similarly, aberrant intermediate filament proteins have been linked to diseases of neurodegeneration (11). Our data clearly show both decrease and abnormalities in MAP-2 expression following treatment with DEET or permethrin or both DEET and permethrin in the brain, particularly the motor cerebral cortex and the CA1 subfield of the hippocampal formation. A decreased expression and beaded appearance of MAP-2 in dendrites would lead to destabilization of dendrites and can result in abnormal functioning of neurons, particularly loss of synapses due to resorption of postsynaptic specializations such as dendritic spines. Such aberrant dendritic organization and the consequently altered connectivity in the cerebral cortex and the hippocampal formation could respectively have profound adverse influence on motor function and learning and memory.

A major component of astrocytic intermediate filament, GFAP is upregulated in response to reactive gliosis as a consequence of a variety of insults, such as exposure to neurotoxic chemicals, trauma, and neurodegenerative diseases that affect the CNS (12). The precise function of GFAP is not well understood, but it is believed to play an important role in the long-term maintenance of brain cytoarchitecture (19), proper functioning of the blood-brain barrier (25), and modulation of neuronal functions (31). Increased expression of GFAP in the soma and processes of astrocytes in various brain regions exhibiting neuronal cell death indicates that neurodegenerative changes induced by exposure to DEET and permethrin, either alone or in combination, are quite robust and lead to a significant hypertrophy of astrocytes. This is because hypertrophied astrocytes (or reactive astrocytes) represent transformed resting astrocytes with increased GFAP accumulation, and this transformation occurs as a consequence of injury to the brain. The accumulation of reactive astrocytes can lead to increased generation of toxic mediators that may cause further pathological damages in the brain (27).

CONCLUSIONS

Most of the earlier studies on the neurotoxic effects of DEET or permethrin used routes of exposure that are not directly germane to the contact exposure, as is believed to have occurred during the Gulf War. The results of this study, however, clearly suggest that

subchronic dermal application of these chemicals leads to diffuse neuronal cell death and significant neuronal cytoskeletal abnormalities in the motor cerebral cortex, the hippocampal formation, and the cerebellum. Taken together, these alterations can lead to many physiological and behavioral abnormalities, particularly motor deficits and learning and memory dysfunction. The above alterations are likely the contributory factors for neurobehavioral abnormalities observed earlier in adult rats following exposure to DEET and permethrin, alone or in combination. Thus, it is likely that subchronic exposure to DEET and permethrin experienced by service personnel during the Persian Gulf War has played an important role in the development of illnesses in some veterans after the Gulf War.

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Induction of urinary excretion of 3-nitrotyrosine, a marker of oxidative stress, following administration of pyridostigmine bromide, DEET (*N,N*-diethyl-*m*-toluamide) and permethrin, alone and in combination in rats

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Abstract

In this study, we determined levels of 3-nitrotyrosine in rat urine following administration of a single oral dose of 13 mg/kg pyridostigmine bromide (PB) (3-dimethylaminocarbonyloxy-*N*-methylpyridinium bromide), a single dermal dose of 400 mg/kg *N,N*-diethyl-*m*-toluamide (DEET) and a single dermal dose of 1.3 mg/kg permethrin, alone and in combination. Urine samples were collected from five treated and five control rats at 4, 8, 16, 24, 48, and 72 h following dosing. Solid-phase extraction coupled with high-performance liquid chromatography with ultraviolet detection at 274 nm was used for the determination of tyrosine and 3-nitrotyrosine. A single oral dose of PB and a single dermal dose of DEET or their combination significantly ($P < 0.05$) increased levels of 3-nitrotyrosine starting 24 h after dosing compared with control urine samples. The maximum increase of 3-nitrotyrosine was detected 48 h after combined administration of PB and DEET. The ratio of 3-nitrotyrosine to tyrosine in urine excreted 48 h after dosing was 0.19 ± 0.04 , 0.20 ± 0.05 , 0.28 ± 0.03 , 0.32 ± 0.04 , 0.19 ± 0.05 , 0.42 ± 0.04 , 0.27 ± 0.03 , 0.36 ± 0.04 , and 0.48 ± 0.04 following administration of water, ethanol, PB, DEET, permethrin, PB + DEET, PB + permethrin, DEET + permethrin, and PB + DEET + permethrin, respectively. The results indicate that an oral dose of PB and a dermal administration of DEET, alone and in combination, could generate free radical species, and thus increase levels of 3-nitrotyrosine in rat urine. Induction of 3-nitrotyrosine, a marker of oxidative stress, following exposure to these compounds could be significant in understanding the proposed enhanced toxicity following combined exposure to these compounds. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Pyridostigmine bromide; *N,N*-Diethyl-*m*-toluamide; Permethrin; 3-Nitrotyrosine; Oxidative stress

1. Introduction

Cells generate nitrogen oxide and other reactive oxygen species such as the superoxide anion. The reaction forms peroxynitrite (Kaur and Halliwell,

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1994; Hensley et al., 2000). Peroxynitrite reacts with tissue protein tyrosine to form 3-nitrotyrosine that is used as a marker for oxidative stress (Roberts et al., 1998). Several reports have been published indicating induction of 3-nitrotyrosine following exposure to chemicals (Salman-Tabchen et al. 1995; Pennathur et al., 1999; Yi et al., 2000).

Several methods have been used for the determination of 3-nitrotyrosine in biological matrices (Herce-Pagliai et al., 1998; Rimbach et al., 1999). Among these methods are high-performance liquid chromatography (HPLC) with UV, photodiode array or electrochemical detection (Salman-Tabchen et al., 1995; Oury et al., 1995; Kamisaki et al., 1996; Fukuyama et al., 1997; Kaur et al., 1998; Tabrizi-Fard et al., 1999; Hirabayashi et al., 1999, 2000; Sodum et al., 2000; Van Dalen et al., 2000).

Pyridostigmine bromide (PB) (3-dimethylaminocarbonyloxy-*N*-methylpyridinium bromide) is a drug used in the treatment of patients of myasthenia gravis, an autoimmune disease affecting the neuromuscular junction and producing weakness of voluntary muscles (Aquilonius and Hartvig, 1986), and is applied as a prophylaxis against nerve agents (Abou-Donia et al., 1996; Young and Evans, 1998). It inhibits acetylcholinesterase and butyrylcholinesterase in the peripheral nervous system (Abou-Donia et al., 1996). Permethrin is an insecticide effective against mites and head lice (Burgess et al., 1992). Permethrin modifies the sodium channel to open longer during a depolarization pulse (Narahashi, 1985). *N,N*-Diethyl-*m*-toluamide (DEET) is applied as an insect repellent (Brown and Hebert, 1997). Previous studies have shown that DEET has a direct effect on the nervous system in laboratory animals, resulting in spongiform myelinopathy in the brain stem with signs including ataxia, seizures, coma and death (Verschoyle et al., 1990). Furthermore, extensive and repeated topical application of DEET resulted in human poisoning including two deaths (Roland et al., 1985; Edwards and Johnson, 1987). The three compounds were used during the Gulf War to protect veterans against nerve agents and biting insects (Young and Evans, 1998). Combined exposure to PB, permethrin and DEET enhanced

neurotoxicity in hens (Abou-Donia et al., 1996) and increased mortality in rats (McCain et al., 1997). Published reports implicated combined exposure to these compounds that resulted in enhanced neurotoxicity in chickens, increased mortality in rats, or induced behavioral changes in the Gulf War Illnesses (Abou-Donia et al., 1996; Haley and Kurt, 1997; Kurt, 1997; Wilson et al., 1998; Hoy et al., 2000).

In this study, we present results of analysis of urine levels of 3-nitrotyrosine following administration of PB, DEET and permethrin, alone and in combination, in rats.

2. Materials and methods

2.1. Chemicals and materials

Tyrosine, 3-nitrotyrosine and pyridostigmine bromide (98% PB) (Fig. 1) were obtained from Sigma Co Inc. (St. Louis, MO). DEET ($\geq 97\%$) was purchased from Aldrich Chemical Co, Inc. (Milwaukee, WIS), while permethrin (99%; 3-(2,2-dichloroethyl))

-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl)methyl ester) was purchased from Chem Service, Inc. (West Chester, PA). Water and acetonitrile (HPLC grade) were obtained from Mallinckrodt Baker, Inc. (Paris, Kentucky). C₁₈ Sep-Pak Cartridges were obtained from Waters Corporation (Milford, MA).

2.2. Animal treatment and use

Sprague-Dawley rats (200–240 g) were purchased from Zivic Miller (Zelienople, PA). The animals were kept in a 12-h light/dark cycle (temperature, 21–23°C) for 1 week, and were provided with a free supply of feed (Rodent Laboratory Chow; Ralston Purine Co., St. Louis, MO) and tap water. Animal care was conducted according to NIH guidelines. The RAC approval number is A612-99-12-2. A single dose of 13 mg/kg PB was applied orally. A single dose of 1.3 mg/kg permethrin and of 400 mg/kg DEET were applied in ethanol with a micropipette to an unprotected 1 cm² area of pre-clipped skin on the back of each

rat. A group of five animals was used for each time point. (The doses represent real-life exposure (Abu-Qare and Abou-Donia, 2000a,b).) A combined single dermal dose of 13 mg/kg pyridostigmine bromide and 1.3 mg/kg permethrin, followed by a single dermal dose of 400 mg/kg DEET was also applied. Five control rats were treated with equal volume of either water or ethanol and kept under similar conditions as treated rats. Urine samples were collected at 4, 8, 16, 24, 48, and 72 h after dosing. The samples were frozen at -20°C until analysis. A 2 ml sample of the collected urine at each time point was taken for analysis.

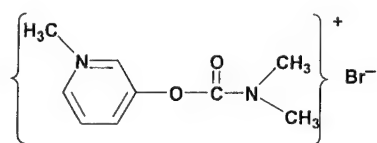
2.3. HPLC system

The liquid chromatographic system (Waters 2690 Separation Module) consisted of a Waters 600E Multisolute delivery system pump, a Waters Ultra WISP 715 autoinjector and a Waters

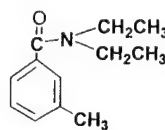
2487 Dual λ absorbance detector (Waters Corporation, Milford, MA). A guard column (Supelco, 2 cm \times 4.0 mm, 5 μm ; Supelco Park, Bellefonte, PA), and a reversed-phase C_{18} column $\mu\text{Bondapak}^{\text{TM}} \text{C}_{18}$, 125 \AA , 10 μm , $3.9 \times 300 \text{ mm}^2$ were used (Waters Corporation, Milford, MA)

2.4. Calibration curve, recovery and limits of detection

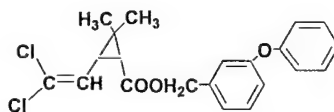
The standard calibration curves for tyrosine and 3-introtyrosine were linear for concentrations of 0.1, 0.2, 0.4, 0.5, 1, and 10 $\mu\text{g/ml}$. The calibration curves were generated using the GRAPH PAD PRISM program for Windows (GraphPad Software Inc., San Diego, CA). Detection limits were determined as the lowest concentration that can be detected taking into consideration a 1:3 base line:peak signal ratio. Recoveries of the compounds from blank urine samples collected at 0 h of the experiment were determined for concentra-



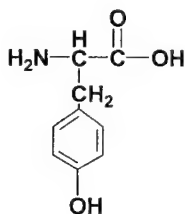
Pyridostigmine bromide



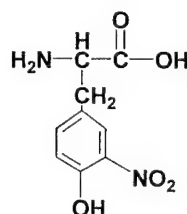
DEET (*N,N*-diethyl-*m*-toluamide)



Permethrin



Tyrosine



3-Nitrotyrosine

Fig. 1. Structures of pyridostigmine bromide, DEET (*N,N*-diethyl-*m*-toluamide) permethrin, tyrosine, and 3-nitrotyrosine.

tions of 0.1–10 µg/ml. Known concentrations were spiked with control urine samples (0 h samples) and analyzed as described under sample preparation. Amounts of tyrosine and 3-nitrotyrosine were corrected based on the recovery obtained.

2.5. Sample preparation

A volume of 2.0 ml urine samples was acidified (pH 5.0) using 0.1 M acetic acid, and then loaded on a disposable C₁₈ Sep-Pak Vac 3 cm³ (500 mg) cartridge (Waters Corporation, Milford, MA) previously conditioned with 2 ml methanol, and equilibrated using 2 ml water and 2 ml of 0.03 M phosphate dibasic buffer (pH 5.0) prior to use. After washing with 2 ml water, the sample was eluted using 2 × 1 ml methanol, and the methanolic eluates were reduced to 500 µl in small test tube using a gentle stream of nitrogen at room temperature, prior to analysis by HPLC. Urine samples were diluted to fit within the calibration curves.

2.6. Analysis of urine samples

A volume of 10.0 µl extracts was injected into the HPLC system as already described. The mobile phase consisted of 84% water (adjusted to pH 3.0 using 0.1 M acetic acid) and 16% methanol at flow rate of 0.60 ml/min. The eluents were monitored by UV detection at dual wavelengths of 274 and 254 nm. The chromatographic analysis was performed at ambient temperature. The amount of tyrosine and 3-nitrotyrosine was calculated and corrected based on the urine volume and total body weight of the animal.

2.7. Statistical analysis

Analysis of variance using a GRAPHPAD PRISM program for Windows (GraphPad Software Inc., San Diego, CA) was used to determine whether the difference between treated and control is significant. A value of $P < 0.05$ was considered

statistically significant. Values are expressed as mean ± S.D. of five replicates.

3. Results

3.1. Linearity, recovery and limits of detection

Calibration standard curves for tyrosine and 3-nitrotyrosine were linear for concentrations ranging between 0.1 and 10 µg/ml, with correlation coefficients $r^2 > 0.998$. The average percentage recoveries of tyrosine and 3-nitrotyrosine ranged from 78.8 to 92.5%. Limit of detection of tyrosine and 3-nitrotyrosine was 0.2 ng (20 ng/ml).

3.2. HPLC analysis

Fig. 2a,b shows chromatograms of standard and urine samples of tyrosine and 3-nitrotyrosine obtained under the described HPLC conditions. Retention times were 4.4 and 7.1 min for tyrosine and 3-nitrotyrosine, respectively.

3.3. Levels of L-tyrosine and 3-nitrotyrosine

A single oral dose of 13 mg/kg PB and a single dermal dose of 400 mg/kg DEET caused significant increase in the urinary excretion of 3-nitrotyrosine when applied, alone or in combination, after 24 h of dosing (Fig. 3). Maximum induction was detected 48 h after dosing. Amounts of tyrosine and 3-nitrotyrosine were corrected according to total volume of the urine and body weight of the animal and the recovery from urine samples.

3.4. Statistical analysis

There was a statistically significant increase in the amount of 3-nitrotyrosine after 24 h of administration of PB, DEET, and their combination ($P < 0.05$). Administration of a single permethrin did not cause a significant effect on the level of 3-nitrotyrosine compared with control urine ($P > 0.05$).

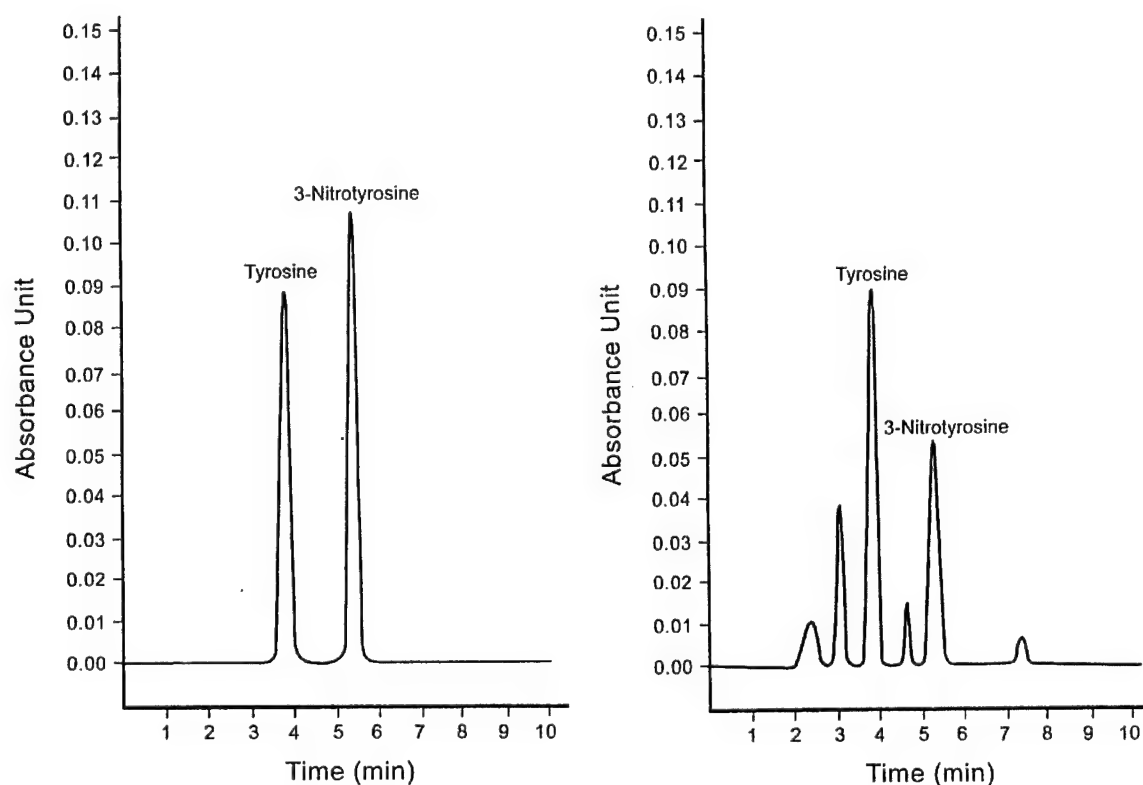


Fig. 2. (a) HPLC chromatogram of standard solutions of tyrosine and 3-nitrotyrosine. (b) HPLC chromatogram of urine samples following a single oral dose of pyridostigmine bromide and a single dermal dose of DEET (*N,N*-diethyl-*m*-toluamide) and permethrin.

4. Discussion

The results indicate that a single oral dose of PB and a single dermal administration of DEET, alone and in combination, significantly increased rat urinary excretion of 3-nitrotyrosine starting 24 h after dosing. In this study, we used a real-life exposure levels as determined by the US Department of Defense (personal communications). Induction of 3-nitrotyrosine has been widely studied as marker of oxidative stress. Several published reports implicated its induction with diseases. A variety of chemicals have been shown to increase its levels in different biological matrices. Pennathur et al. (1999) reported that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine increased levels of 3-nitrotyrosine in brain tissues. Furthermore, Imam et al. (1999) reported that administration of metham-

phetamine in mice resulted in the significant formation of 3-nitrotyrosine in the striatum. It is believed that formation of 3-nitrotyrosine proceeds through free radical mechanism (Pannala et al., 1998; Niwa et al., 1999). It might be that PB and DEET induced generation of free radicals, and thus formation of peroxynitrite, and then increased 3-nitrotyrosine. Involvement of free radicals in the formation of 3-nitrotyrosine was detected following irradiation of *S*-nitrosoglutathione in the presence of tyrosine. Pannala et al. (1998) reported that hydroxycinnamates antioxidants decreased peroxynitrite-mediated nitration of tyrosine in vitro. Furthermore, Niwa et al. (1999) showed that the antioxidants caffeic acid and sinapinic acid inhibited formation of 3-nitrotyrosine in protein treated with peroxynitrite. An increased concentration of 3-nitrotyrosine has been implicated in various diseases that resulted

in oxidative stress conditions (Halliwell et al., 1999; Gole et al., 2000; Schwemmer et al., 2000). An increased plasma level of 3-nitrotyrosine was considered as a biomarker of oxidative stress in chronic renal failure patients with septic shock (Fukuyama et al., 1997). Furthermore, Kaur and Halliwell (1994) reported that blood serum from patients with the inflammatory joint disease rheumatoid arthritis contained 3-nitrotyrosine, while there was no detection of 3-nitrotyrosine in blood serum of normal subjects. Tohgi et al. (1999) reported an increase by more than sixfold

in the level of 3-nitrotyrosine and of the 3-nitrotyrosine/tyrosine ratio in neurons of patients with Alzheimer's disease compared with normal controls of similar age.

The results show that exposure of rats to a single dermal dose of 1.3 mg/kg permethrin did not cause significant increase of urinary excretion of 3-nitrotyrosine, suggesting that oxidative stress was similar to that of control levels, while an oral dose of PB and a dermal dose of DEET, alone or in combination, significantly increased levels of 3-nitrotyrosine in rat urine.

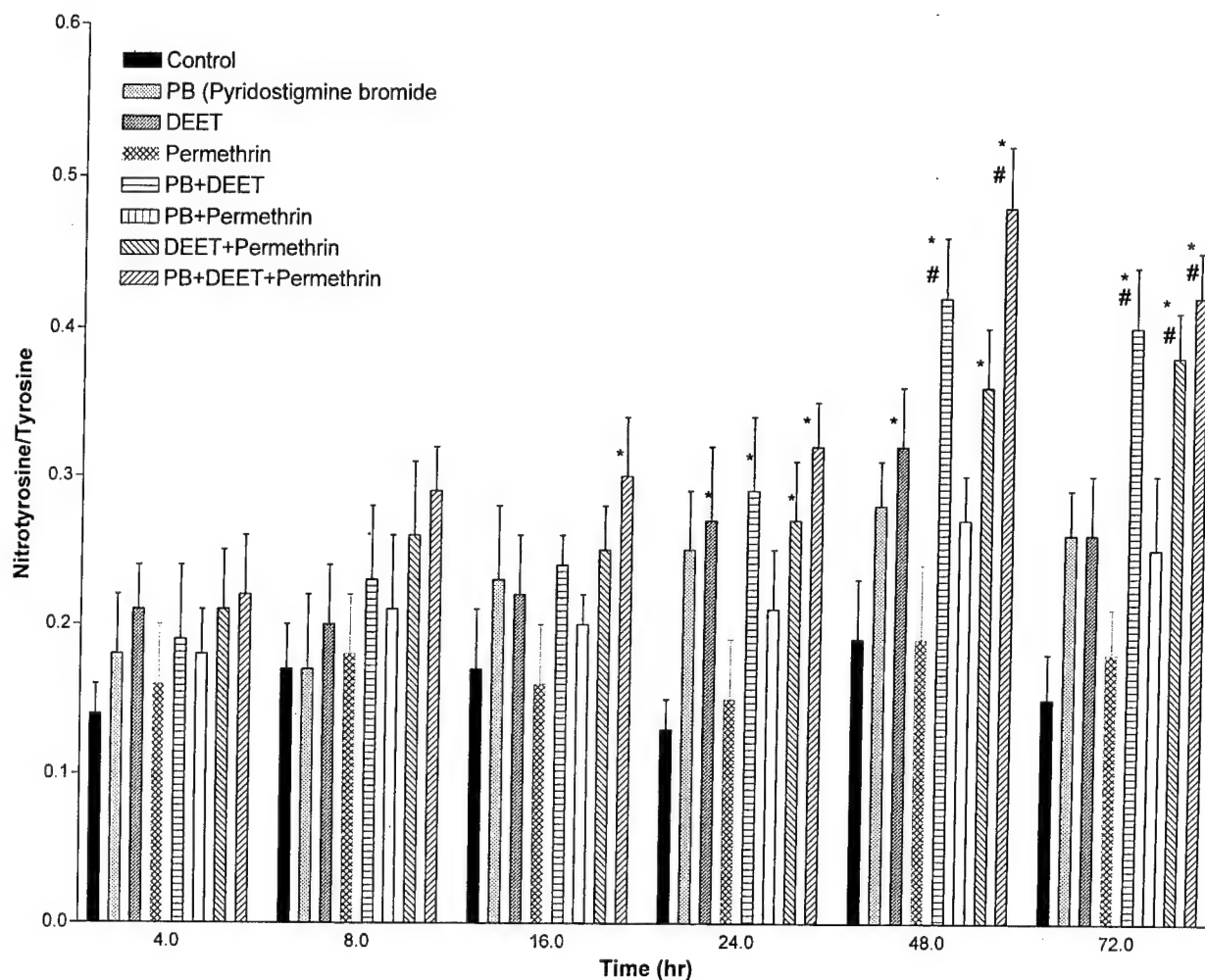


Fig. 3. Ratio of 3-nitrotyrosine to tyrosine in urine samples collected following a single oral dose of pyridostigmine bromide and a single dermal dose of DEET (*N,N*-diethyl-*m*-toluamide) and permethrin, alone and in combination. The ratio of 3-nitrotyrosine to tyrosine in control urine samples collected at 0 h was 0.15 ± 0.04 . * Significantly different from controls at $P < 0.05$. # Significantly different from treated with individual compound at $P < 0.05$.

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COMBINED EXPOSURE TO DEET (*N,N*-DIETHYL-*m*-TOLUAMIDE) AND PERMETHRIN-INDUCED RELEASE OF RAT BRAIN MITOCHONDRIAL CYTOCHROME *c*

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The release of cytochrome c from the mitochondrial intermembrane space can induce apoptosis. The levels of mitochondrial cytochrome c in rat brain following a single dermal dose of 400 mg/kg of DEET, and of 1.3 mg/kg of permethrin, alone or in combination were determined. Rats were sacrificed at a time interval of 0.5, 1, 2, 4, 8, 16, 24, 48, or 72 h after dosing. Brain mitochondria were isolated and the levels of cytochrome c were measured using reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. Average percentage recovery of cytochrome c spiked with control rat brain mitochondria was $83.2 \pm 8.9\%$. Limits of detection and quantitation were 1 and 5 ng, respectively. The results showed that a single dermal dose of a combination of DEET and permethrin significantly increased the release of brain mitochondrial cytochrome c starting 24 h after treatment. DEET and permethrin alone did not affect the release of cytochrome c. The results indicate that combined exposure to DEET and permethrin might induce the apoptotic processes in rat brain as seen by the release of cytochrome c.

Generation of reactive oxygen species could lead to an early release of mitochondrial cytochrome *c* that is associated with inhibition of mitochondrial respiration and stimulation mitochondrial superoxide production (Cai & Jones, 1998; Anson et al., 2000). Further, the release of cytochrome *c* from mitochondria as a result of cell death leads to caspase activation (Abu-Qare & Abou-Donia, 2000a; Kuwana et al., 1998). Cells undergoing apoptosis exhibit an elevation of cytochrome *c* in the cytosol and a corresponding decrease in the mitochondria (Yang et al., 1997; Bobba et al., 1999; Finucane et al., 1999; Steemans et al., 1998; Balk et al., 1999). Bossy-Wetzel et al. (1998) reported that cytochrome *c* release from mitochondria is an early event in the apoptotic process induced by ultraviolet B (UVB) irradiation or saturosporine treatment in CEM or HeLa cells. In a cell-free apoptotic system, mitochondria were shown to spontaneously release cytochrome *c* (Kluck et al., 1997). Further, Schuler et al. (2000) reported that in vitro p53 tumor suppressor gene activates the apoptotic

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machinery through induction of the release of cytochrome *c* from mitochondrial intermembrane space, and that cell death was accompanied by caspase activation and cleavage of caspase substrates. Gorman et al. (1999) demonstrated a close correlation between the time course of cytochrome *c* release from the mitochondria and caspase-3 activation during colchicine-induced apoptosis of cerebellar granule cells, while Finucane et al. (1999) indicated that the mitochondrial release of cytochrome *c* represents a critical step in cell death.

Levels of cytochrome *c* in cells and cellular tissues have been determined by the immunoblotting techniques, using a monoclonal antibody to cytochrome *c* (Zhuang & Cohen, 1998; Denecker et al., 2000; Fujii et al., 2000). High-performance liquid chromatography (HPLC) techniques with UV detection at 393 nm have been recently used for determination of mitochondrial cytochrome *c* (Picklo et al., 1999). This method has advantages over the classical immunoblotting method, which has shortcomings such as (1) the quantification of an antigen by immunoblot is limited technically, (2) it may be complicated by incomplete transfer of the antigen to the blotting membrane, (3) masking of the antigen by cellular protein, (4) overdevelopment or underdevelopment of the blot, leading to nonlinearity of the signal, and (5) impracticality of a large number of samples (Picklo et al., 1999).

Permethrin is a pyrethroid insecticide effective against mites, such as head lice (Fraser, 1994). DEET is applied on the skin as an insect repellent against mosquitoes, sand flies, ticks, and fleas (Brown & Hebert, 1997; Davis, 1985). Both compounds are widely used inside homes and in public places (Burgess et al., 1992).

Permethrin modifies sodium channels to open longer during a depolarization pulse (Narahashi, 1985). Previous studies showed that DEET has direct adverse effects on the nervous system in laboratory animals, resulting in spongiform myelinopathy in the brainstem with signs including ataxia, seizures, coma, and death (Verschoyle et al., 1990). Further, extensive and repeated topical application of DEET resulted in human poisoning including two deaths (Edwards & Johnson, 1987; Roland et al., 1985). Combined exposure to permethrin and DEET enhanced neurotoxicity in hens (Abou-Donia et al., 1996), induced urinary excretion of 8-hydroxy-2-deoxyguanosine in rats (Abu-Qare & Abou-Donia, 2000b), and increased mortality in rats (McCain et al., 1997). Published reports implicated exposure to DEET and permethrin in the Gulf War veterans' illnesses (Abou-Donia et al., 1996; Haley et al., 1999; Haley & Kurt, 1997; Hodgson & Kipen, 1999; Hoy et al., 2000).

No published reports have examined possible induction by DEET and permethrin, alone or in combination, of the release of cytochrome *c* into cytosol. In this study, the release of rat brain mitochondrial cytochrome *c* was determined after a single dermal dose of DEET and of permethrin, alone and in combination, using reversed-phase high-performance liquid chromatography method with UV detection at 393 nm.

MATERIALS AND METHODS

Chemicals

Rat heart cytochrome *c* (97%), and phenylmethylsulfonyl fluoride (>99%) were obtained from Sigma Co., Inc. (St. Louis, MO). DEET ($\geq 97\%$, *N,N*-diethyl-*m*-toluamide) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI), while permethrin [99%, 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid (3-phenoxyphenyl)methyl ester] was obtained from Chem Service, Inc. (West Chester, PA). Water and acetonitrile (HPLC grade) were obtained from Mallinckrodt Baker, Inc. (Paris, KY).

HPLC System

The liquid chromatographic system (Waters 2690 separation module) consisted of a Waters 600E multisolvent delivery system pump, a Waters Ultra WISP 715 autoinjector, and a Waters 2487 dual- λ absorbance detector (Waters Corporation, Milford, MA). A guard column (Supelco, 2 cm \times 4 mm, 5 μ m, Supelco Park, Bellefonte, PA) and a reversed-phase C_{18} column, μ Bondapak C_{18} 10 μ m, 3.9 \times 300 mm, were used (Waters Corporation, Milford, MA).

Calibration Curve, Recovery, and Limits of Detection

Standard calibration curves for concentrations between 0.5 and 50 μ g/ml of cytochrome *c* were obtained under the described HPLC conditions. The detection limits were determined as the lowest concentration that can be detected, taking into consideration a 1:3 baseline:peak signal ratio. Recoveries of cytochrome *c* from control rat brain mitochondria samples were determined for concentrations of 0.5–50 μ g/ml.

Experimental Animals

Sprague-Dawley rats (250–300 g) were purchased from Zivic Miller (Zelienople, PA). The untreated animals were kept in a 12-h light/dark cycle (temperature 21–23°C) and were provided with a free supply of feed (rodent laboratory chow, Ralston Purina Co., St. Louis, MO) as well as tap water. Animal care was conducted according to institutional guidelines.

Animal Treatment

Permethrin and DEET were dissolved in 70% ethanol. A single dose of 1.3 mg/kg of permethrin and a single dose of 400 mg/kg of DEET were applied with a micropipette (1 ml/kg) to an unprotected 1-cm² area of preclipped skin on the back of each rat. A group of five animals was used for each time point. A combined single dermal dose of 1.3 mg/kg of permethrin followed by a single dermal dose of 400 mg of DEET was also applied. The dosage selected represents real-life exposure of each chemical as determined by U.S. Department of Defense (personal communications). Control animals were treated with equal volume of 70% ethanol (1 ml/kg) and kept under conditions similar to treated rats. Five animals were anesthetized with halothane (Halocarbon Laboratories, River Edge, NJ)

and sacrificed by heart exsanguination at time intervals of 0.5, 1, 2, 4, 8, 16, 24, 48, or 72 h after dosing. Rat brain was removed, blotted dry and washed in saline, then stored at -70°C prior to analysis by HPLC.

Isolation of Brain Mitochondrial Cytochrome c

Mitochondria samples from rat brain were isolated using a modified method of Clark and Nicklas (1970). Rat brain (500 mg) was homogenized in 4 volumes of buffer [0.32 M sucrose, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 10 $\mu\text{g}/\text{ml}$ leupeptin] in a Potter-Elvehjem homogenizer at 4°C . The homogenate was centrifuged at $1000 \times g$ for 20 min and the supernatant was centrifuged at $12,000 \times g$ for 25 min at 4°C . The resulting supernatant was centrifuged again at $100,000 g$ for 30 min to assure removal of all mitochondria. The resulting supernatant (cytosolic fraction) was then injected into the HPLC system for analysis of the release of cytochrome c into the cytosol. Protein content was estimated by the procedure of Smith et al. (1985).

Analysis

A volume of 10 μl of the extracts was injected into the HPLC system as already described. The mobile phase consisting of 0.1% (v/v) trifluoroacetic acid in water (pH 4.5) (solvent A) and acetonitrile (solvent B) was delivered at a flow rate of 1 ml/min with a linear increase of solvent B from 20% to 60% within 8 min. The column was washed after each run with 60% acetonitrile in 0.1% fluoroacetic acid for 5 min, then equilibrated with the mobile phase for 3 min. The eluents were monitored by detection at 393 nm. The chromatographic analysis was performed at ambient temperature.

Statistical Analysis

Analysis of variance (ANOVA) using the GraphPad Prism program for Windows (GraphPad Software, Inc., San Diego, CA) was used to determine if the difference between treated and control was statistically significant; $p < .05$ was considered statistically different.

RESULTS

Linearity, Recovery, and Detection Limits

A calibration standard curve for cytochrome c was obtained for a concentration range between 0.5 and 50 $\mu\text{g}/\text{ml}$ (5–500 ng). Percentage average recovery of cytochrome c from rat brain mitochondria was $83.2 \pm 8.9\%$. Limits of detection and quantitation of rat cytochrome c were 1 ng and 5 ng, respectively.

HPLC Analysis

Figures 1a and 1b represent the chromatograms of standard and rat brain mitochondrial cytochrome c under described HPLC conditions. Retention time of cytochrome c was 9.2 min.

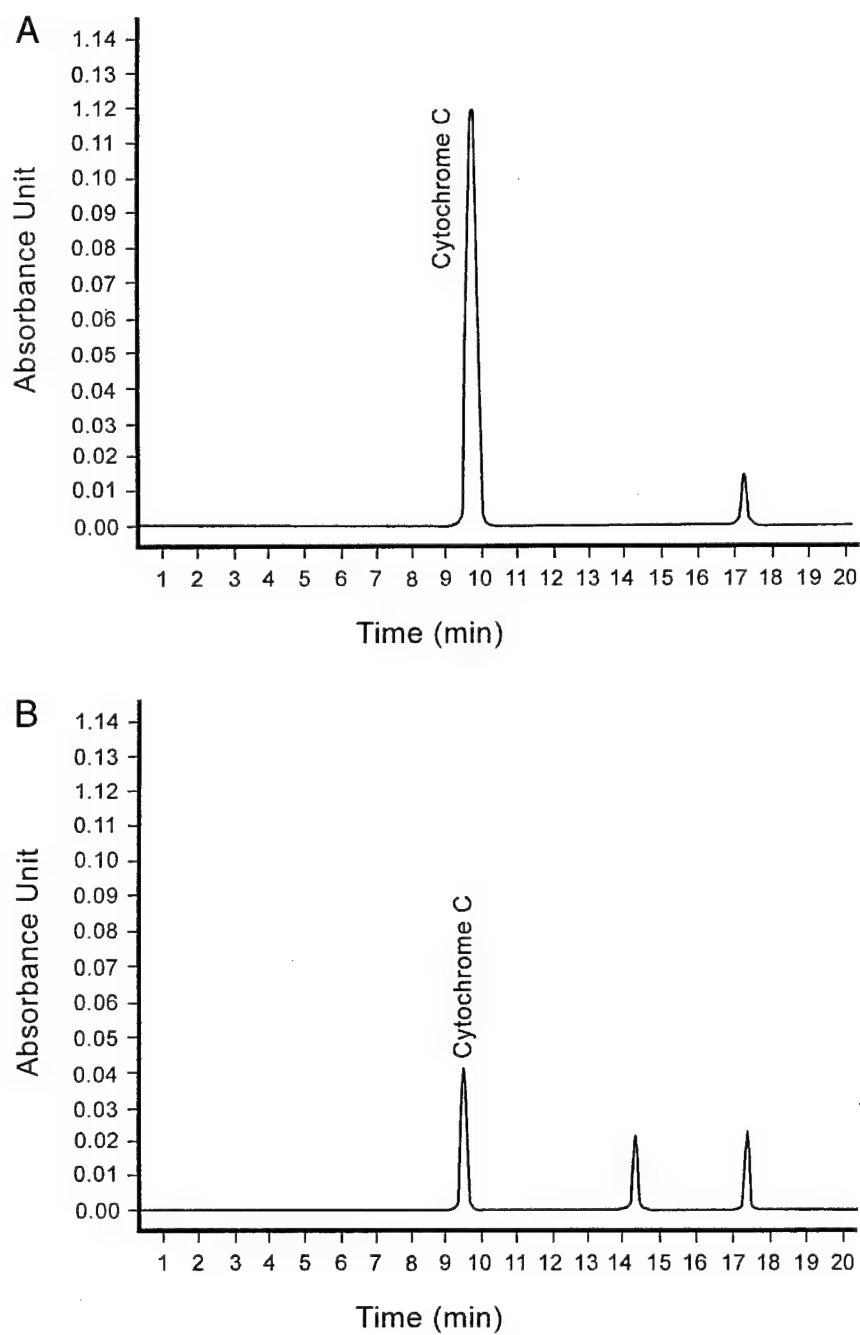


FIGURE 1. (a) HPLC chromatogram of cytochrome c standard. (b) HPLC chromatogram of cytosolic fraction of rat brain treated with a combined dose of DEET and permethrin.

Levels of Cytochrome c in Control and Treated Rats

Combined exposure to a single dermal dose of DEET and permethrin caused significant increase in the release of mitochondria cytochrome c 24 h after dosing (Figure 2). A single dermal dose of DEET or permethrin did not produce statistically significant effect on the release of cytochrome c from brain mitochondria (Figure 2) over the time course of treatment. The mean (\pm SD) level of the released rat brain mitochondrial cytochrome c was 0.69 ± 0.1 , 1.1 ± 0.1 , 0.71 ± 0.06 , and 1.7 ± 0.08 μ g/mg mitochondria protein at 24 h after dosing of ethanol, DEET, permethrin, and a combination of DEET and permethrin, respectively.

A combined dose of DEET and permethrin caused significant induction of cytochrome c compared to control starting at 24 h until 72 h after dosing (Figure 2). On the other hand, there was no significant difference between DEET or permethrin treatment and ethanol-treated rats.

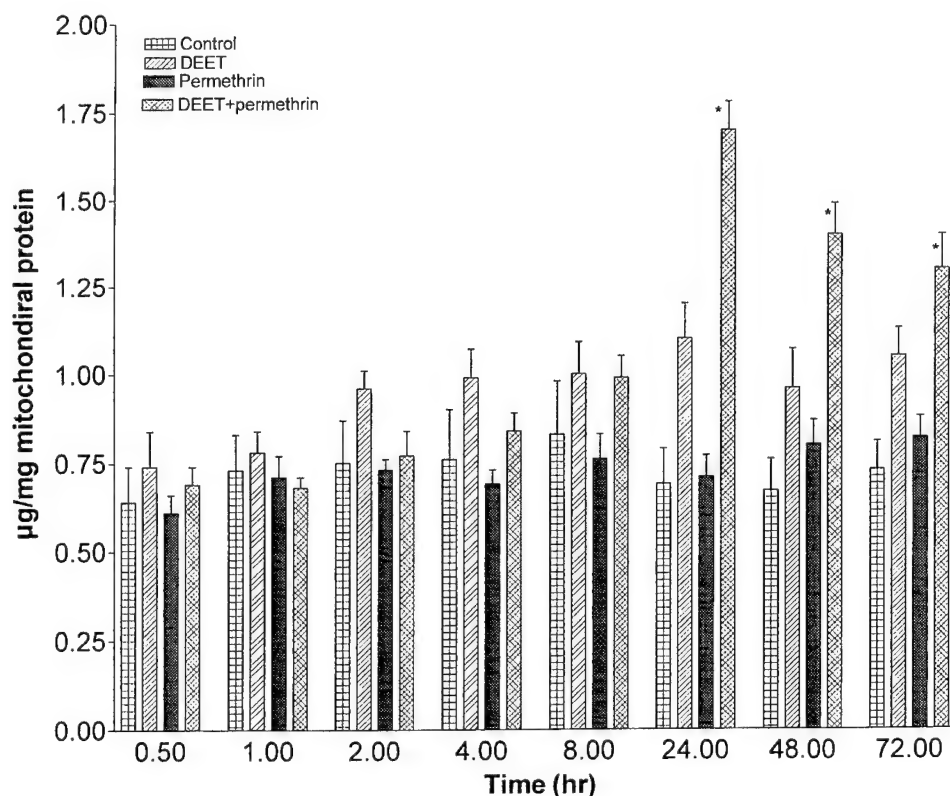


FIGURE 1. Levels of rat brain cytosol cytochrome c (μ g/mg mitochondrial protein) following a single dermal dose of 400 mg/kg of DEET and 1.3 mg/kg of permethrin, alone and in combination. Asterisk indicates significantly different from control, $p < .05$.

DISCUSSION

Our results indicate that combined dermal administration of DEET and permethrin to rats significantly induced the release of rat brain mitochondrial cytochrome c starting 24 h after dosing. In previous studies, the release of cytochrome c has been correlated with induction of the apoptotic processes. Martino et al. (2000) and Bossy-Wetzel et al. (1999) reported that during the process of apoptosis, cytochrome c is released from mitochondria into the cytosol, where it helps to activate the caspases, a family of killer proteases. Heiskanen et al. (1999) detected the release of cytochrome c from mitochondria into cytosol in cells undergoing apoptosis. Further, the release of cytochrome c was rapid and complete during hypoxia/reoxygenation of cultured kidney cells, resulting in caspase activation, whereas cells that lack cytochrome c underwent apoptosis after reoxygenation (Saikumar et al. (1998). Kannan et al. (2000) reported that the organochlorine insecticide endosulfan induced the release of cytochrome c in human T-leukemic cell lines. Factors that inhibit or block the release of cytochrome c protect the cells from apoptosis. Kharbanda et al. (1997) reported that Bcl-xL inhibited the accumulation of cytosolic cytochrome c, and prevented cell apoptosis in response to genetic stress. Our results are in agreement with previous studies that reported that chemicals induced release of cytochrome c-dependent mechanisms. In vitro incubation of purified mitochondria with pyrrolidine dithiocarbamate directly induced cytochrome c release from the intermembrane space (Della Ragione et al., 2000). Mirkes and Little (2000) showed that the teratogen 4-hydroperoxycyclophosphamide and heat shock induced the release of cytochrome c from mitochondria in 9-d-old mouse embryos. Further, exposure to phosphatidyl serine caused the release of cytochrome c and decreased transmembrane potential in cells (Denecker et al., 2000). Li et al. (1999) also reported that apoptotic cell death induced by beta-lapachone, a potential anticancer drug, was preceded by a rapid release of cytochrome c, followed by activation of caspase 3 in apoptotic cell death. Furthermore, treatment of HL-60 cells with staurosporine (STS) induced mitochondrial cytochrome c efflux into the cytosol, which was followed by caspase-3 activation and apoptosis (Han et al., 1998).

Environmental factors and stress play a role in the release of cytochrome c. Balk et al. (1999) showed that heat (55°C) induced programmed cell death in cucumber plants through translocation of cytochrome c from mitochondria to cytosol. Further, Chen et al. (2000) reported that ionizing radiation induced rapid release of cytochrome c from mitochondria into the cytoplasm, which activated caspases, and resulted in the breakdown of the amino acid sequence DEVD that was involved in apoptosis. Exposure of human cervical carcinoma C33A cells to the anticancer drug *N*-(4-hydroxyphenyl) retinamide induced cytochrome c release from mitochondria to cytoplasm, increased reactive oxygen species (ROS), activated

caspase-3, and caused transition membrane permeability (Suzuki et al., 1999).

Significant induction of the release of cytochrome c has been detected 24 h after combined exposure to DEET and permethrin. The lack of an induction of the release of cytochrome c at early times following administration suggests slow absorption of the test compounds that resulted in concentrations below the threshold levels of cytochrome c release. In this study, the dose of DEET and permethrin was at a real-life exposure level as determined by U.S. Department of Defense (personal communications).

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Binding of pyridostigmine bromide, *N,N*-diethyl-*m*-toluamide and permethrin, alone and in combinations, to human serum albumin

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Abstract In this study we examined the interaction of the anti-nerve agent drug pyridostigmine bromide (PB, 3,3-dimethylaminocarbonyloxy-*N*-methylpyridinium bromide), the insect repellent DEET (*N,N*-diethyl-*m*-toluamide), and the insecticide permethrin [3-(2,2-dichloroethyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl)methyl ester] in binding to human serum albumin (HSA). Concentrations between 500 ng/ml and 10 µg/ml PB, DEET and permethrin, alone or in combination, were incubated with HSA at 37°C for 60 min. Concentrations of PB, DEET and permethrin were determined using high performance liquid chromatography (HPLC). The results showed that $81.2 \pm 4.2\%$, and $84.6 \pm 2.5\%$ of the initial concentration of PB was bound to HSA when incubated alone or in combination with DEET or permethrin, respectively. DEET and permethrin did not significantly interact with HSA after 1 h of incubation. Incubation of combinations of two or three compounds did not significantly alter the binding pattern of any of the compounds with HSA. These results showed that PB is highly bound to albumin protein, while the competition between PB, DEET and permethrin on binding sites of HSA as a possible site of interaction following combined administration in vivo is not likely.

Keywords Pyridostigmine bromide · *N,N*-Diethyl-*m*-toluamide · Permethrin · Human serum albumin · Gulf War illness

Introduction

Pyridostigmine bromide (PB, 3,3-dimethylaminocarbonyloxy-*N*-methylpyridinium bromide) is used for treatment of myasthenia gravis (Flacke 1973; Breyer-Pfaff et al. 1985; Aquilonius and Hartvig 1986), and as prophylactic drug to protect against nerve agents (Keeler et al. 1991; Young and Evans 1998). PB inhibited acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) enzymes in hens and rats (Abou-Donia et al. 1996), and BuChE in mice (Somani et al. 2000). DEET (*N,N*-diethyl-*m*-toluamide) is applied as an insect repellent (Robinson and Cherniak 1986; Brown and Hebert 1997). Previous studies have shown that, in laboratory animals, DEET has direct effect on the nervous system that causes spongiform myelinopathy in the brain stem with signs of ataxia, seizures, coma and death (Verschoyle et al. 1990). Furthermore, extensive and repeated topical application of DEET resulted in human poisoning, including two deaths (Roland et al. 1985; Edwards and Johnson 1987). Permethrin is an insecticide effective in the control of mites and head lice (Miller 1989; Burgess et al. 1992; Fraser 1994). It modifies the sodium channel to open longer during a depolarization pulse (Narahashi 1985). Combined exposure to PB, DEET and permethrin enhanced neurotoxicity in hens and rats (Abou-Donia et al. 1996), increased mortality in rats (McCain et al. 1997), produced behavioral alterations in male rats (Abou-Donia 2001; Hoy et al. 2000), and increased urinary excretion of 3-nitrotyrosine, a marker of oxidative stress in rats (Abu-Qare et al. 2001). Published reports implicated exposure to PB, DEET and permethrin in Veterans Gulf War illnesses (Abou-Donia et al. 1996; Olson et al. 1998; Kurt 1998; Shen 1998; Wilson et al. 1998).

Pharmacokinetics and metabolism of these compounds as individuals have been studied in humans and in animals (Somani et al. 1972; Cohan et al. 1976; Bloomquist and Thorsell 1977; Chan and Calvey 1978; Barber et al. 1979; Aquilonius et al. 1980; Anadon et al. 1991; Taylor et al. 1991; Selim et al. 1995;

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Schoenig et al. 1996). PB is metabolized mainly through hydrolysis to *N*-methyl-3-hydroxypyridinium following administration in rats and humans (Birtley et al. 1966; Taylor et al. 1991; Abu-Qare and Abou-Donia 2000), while metabolism of DEET involves initial oxidative *N*-deethylation, ring hydroxylation, hydrolysis, and conjugation (Taylor 1986; Taylor et al. 1994; Constantino and Iley 1999). Vulule et al (1999) reported that permethrin metabolism was catalyzed by oxidase and esterase enzymes.

Chemical-protein binding is an important process in determining the overall distribution, excretion, activity and toxicity of a chemical and can be an important source of chemical-chemical interaction. The binding could be with a general ligand such as human serum albumin (HSA) or α -acid glycoprotein (Hage and Tweed 1997). Most chemicals bind or complex with proteins by a reversible process (Sharger and Yu 1992).

Several methods have been used to study chemical-protein binding. These methods included high performance liquid chromatography (HPLC) (Domenici et al. 1991; Rahim and Aubry 1995; Ashton et al. 1996; Yang and Hage 1997; Wang et al. 1998; Zhivkova and Russ-eva 1998a; Black et al. 1999), high-performance capillary electrophoresis/frontal analysis (Shibukawa et al. 1994; Zhivkova and Russeva 1998b), gas chromatography (Abdel-Rehim et al. 2000), and spectrophotometric techniques (Parikh et al. 2000).

Several studies reported possible interactions between PB, DEET and permethrin that could enhance their toxicity following combined exposure. No published reports have examined binding of these compounds with human serum proteins. The aims of this study were to examine binding of PB, DEET and permethrin with human serum albumin, and to study possible binding interactions between these compounds.

Materials and methods

Chemicals and materials

Permethrin (99%) was purchased from Chem Service, Inc. (West Chester, Pa., USA). Pyridostigmine bromide (PB, 3,3-dimethylaminocarbonyloxy-*N*-methylpyridinium bromide) (Fig. 1), and human serum albumin (HSA) (99%) were obtained from Sigma Chemical Co., Inc. (St. Louis, Mo., USA). DEET ($\geq 97\%$, *N,N*-diethyl-*m*-toluamide) was obtained from Aldrich Chemical Co, Inc (Milwaukee, Wis., USA). Acetonitrile and water (HPLC grade) were obtained from Mallinckrodt Baker, Inc. (Paris, Ky., USA). C_{18} Sep-Pak cartridges were obtained from Waters Corporation (Waters Corporation, Milford, Mass., USA).

HPLC system

The liquid chromatographic system (Waters 2690 Separation Module), consisted of a Waters 600 E Multisolvant delivery system pumps, a Waters Ultra WISP 715 autoinjector and a Waters 2487 Dual λ absorbance detector (Waters Corporation). A guard column (2 cm \times 4.0 mm, 5 μ m; Supelco, Bellefonte, Pa., USA), and a reversed-phase C_{18} column μ -Bondapak C_{18} 125A (10 μ m, 3.9 \times 300 mm; Waters Corporation) were used.

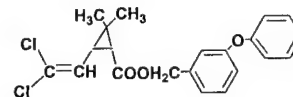
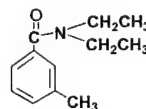
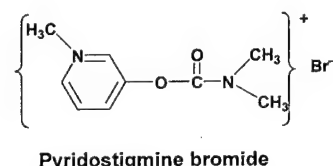


Fig. 1. Structures of pyridostigmine bromide, DEET and permethrin

Chromatographic conditions

The method used was according to previously published method (Abu-Qare and Abou-Donia 2000). The mobile phase was a water (adjusted to pH 3.00 using 0.1 N acetic acid):acetonitrile gradient at a flow rate of 1–1.7 ml/min. The gradient started at 1% acetonitrile, increased to 25% acetonitrile at 3.6 min, then to 45% by 6 min, and up to 85% at 11 min. The system returned to 1% acetonitrile at 14 min and was kept under these conditions for 3 min to re-equilibrate. Total run time was 17 min. The eluents were monitored by UV detection at a range of 210–280 nm. The chromatographic analysis was performed at room temperature.

Calibration procedures, detection limits and recovery

Five different calibration standards were prepared by making different concentrations of PB, DEET and permethrin between 100 and 1000 ng/ml. Linear calibration curves were obtained by plotting the peak areas of the individual chemicals as a function of concentration. The standard curves were used to determine recovery of the chemicals from human serum albumin. For the recovery study, five different concentrations ranged between 100 and 1000 ng/ml were spiked in 1 ml HSA solution, the solution was acidified, extracted and purified as described below prior to analysis by HPLC. Limits of detection were determined as the lowest concentration that can be detected, taking in consideration a 1:3 baseline noise:calibration point ratio.

Incubation

HSA standard solution was prepared in phosphate buffer (pH 7.4). A solution of PB, DEET or permethrin in methanol (50–250 μ l), alone or in combination, was added to 1 ml fresh standard of 40 mg/ml HSA to obtain concentrations of 500 ng/ml to 10 μ g/ml HSA. The composition of the control experiment comprised similar concentrations of PB, DEET, and permethrin in buffer solution (pH 7.4) without HSA. The control mixture and the samples were incubated in a shaking water bath at 37°C for 60 min. The incubated solutions were removed and the volume adjusted to 2 ml using acetonitrile. The precipitated protein was removed by centrifugation at 5,000 g and the supernatant was separated by ultra-filtration. The concentration of the free unbound compound(s) was determined by HPLC. Blank samples of HSA were incubated under similar conditions without addition of the substrate(s). The blank solution was used to determine whether there was interference with the test compounds.

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine whether binding of PB, DEET and permethrin to HSA was

significant. ANOVA was also used to determine whether the difference in binding of each compound, alone or in combination, to HSA was significant. A value of $P < 0.05$ was considered statistically significant. The Prism program for Windows (GraphPad Software, Inc., San Diego, Calif., USA) was used for the analysis.

Results

Recovery and detection limits of PB, DEET and permethrin

PB, DEET and permethrin were separated from human serum albumin incubates using HPLC (Figs. 2 and 3). The percentage recoveries of PB, DEET and permethrin from spiked HSA solutions were $71.9 \pm 7.3\%$, $83.6 \pm 8.7\%$ and $77.8 \pm 9.6\%$ (means \pm SD), respectively. Limits of detection were calculated from a peak signal-to-noise ratio of 3:1. The resulting detection limits were 100 ng/ml for PB, 50 ng/ml for DEET and 20 ng/ml for permethrin.

Binding of PB, DEET and permethrin to HSA

Following incubation with HSA, the average percentages of the initial dose of PB, DEET and permethrin were determined to be $18.9 \pm 4.6\%$, $95.4 \pm 2.8\%$ and $92.6 \pm 3.2\%$, respectively (Fig. 4). The results were corrected according to recovery of each chemical from spiked HSA

Interactions of PB, DEET and permethrin with HSA

Incubation of combinations of PB, DEET and permethrin with HSA did not significantly alter the pattern or extent

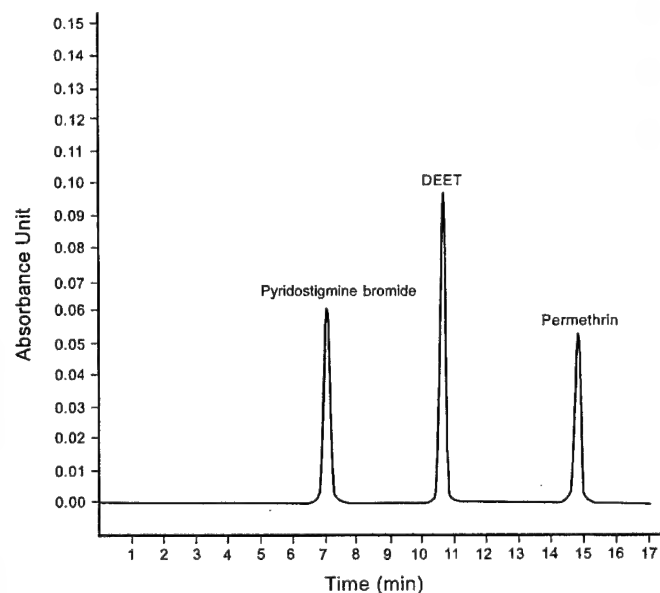


Fig. 2. A representative high performance liquid chromatogram of standards of pyridostigmine bromide, DEET (*N,N*-diethyl-*m*-toluamide) and permethrin under the HPLC conditions described

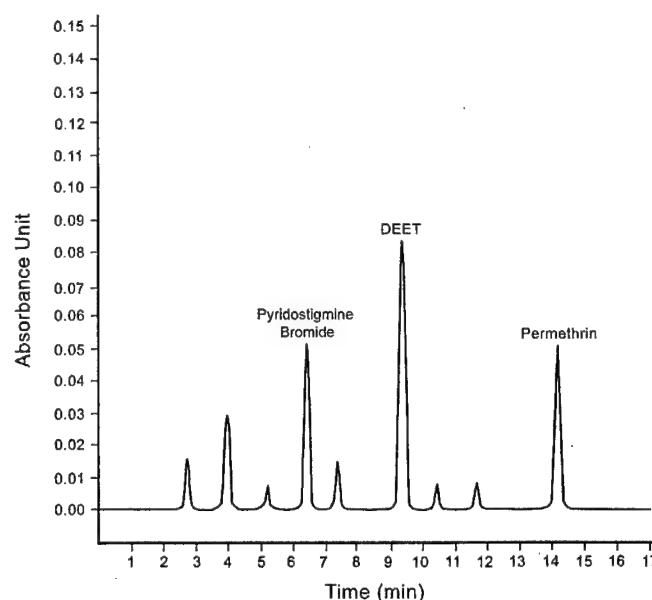


Fig. 3. A representative high performance liquid chromatogram of solution of pyridostigmine bromide, DEET (*N,N*-diethyl-*m*-toluamide) and permethrin following incubation with human serum albumin under the HPLC conditions described

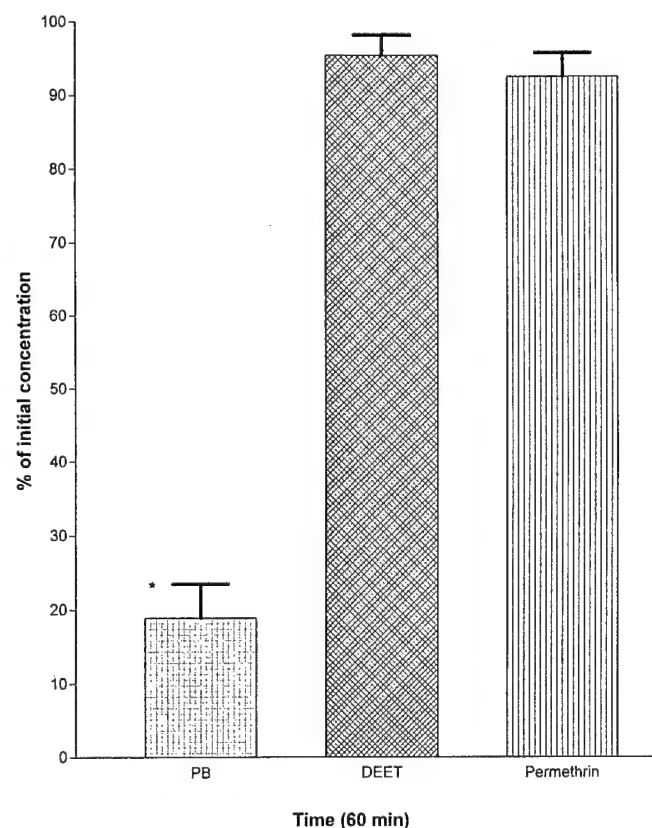


Fig. 4. Levels of pyridostigmine bromide (PB), DEET (*N,N*-diethyl-*m*-toluamide) and permethrin following incubation with human serum albumin. The results are expressed as mean \pm SD of ten samples from five experiments. * $P < 0.01$; the binding is considered statistically significant if $P < 0.05$

of binding to HSA of any individual compound. The concentrations were $16.1 \pm 2.3\%$, $89.3 \pm 1.9\%$ and $91.2 \pm 4.3\%$ of the initial dose for PB, DEET and permethrin, respectively (Fig. 5). The results were corrected according to recovery of each chemical from spiked HSA.

Discussion

In this study, we present a profile interaction of pyridostigmine bromide, DEET and permethrin, alone and in combination, with human serum albumin. The findings have shown that PB significantly bound to albumin protein, which might hinder its immediate absorption and distribution into the circulation system in vivo. In this study, neither DEET nor permethrin significantly bound to HSA. This indicates that both compounds are mainly present as the free form in the serum following absorption into the circulation system. The extent of binding, or the free amount available in the circulating system is one of the factors determining the efficacy of a chemical. In previous reports, chemicals binding to serum proteins have been shown to reduce the free plasma concentrations of a number of anticancer drugs (Finlay

and Baguley 2000). Shibukawa et al. (1995) showed that the anti-diabetic agent troglitazone strongly bound to human serum albumin. Furthermore, Kumar et al. (2000) reported that valproic acid is bound to sheep maternal and fetal plasma, which might affect its efficacy. In this study, neither DEET nor permethrin altered the pattern of PB binding to HSA. This is significant in light of attempts to understand possible pathways of interactions of these compounds that have enhanced toxicity following combined exposure in rats (McCain et al. 1997) and in hens (Abou-Donia et al. 1996). Since PB is administered either to veterans or to myasthenia gravis patients, the possibility of interaction with other drugs has to be considered. In a previous study, Rahim and Aubry (1995) reported that interference between non-steroidal anti-inflammatory drugs and co-administered drugs might due to their interactions at a binding site on HSA. Plum et al. (2000) reported that the binding of the novel oral hypoglycemic agent repaglinide in vitro to human serum albumin has potential clinical significance in that both tolbutamide and furosemide significantly reduced in vitro binding of repaglinide compared with control.

The negligible binding to albumin protein for DEET and permethrin might indicate that binding could occur with other proteins. In a previous study, Bailey (1995) reported that cocaine and cocaethylene had high affinity binding with α_1 -acid glycoprotein and low affinity binding with albumin. Propranolol and verapamil were found to be bound to human α_1 -acid glycoprotein, rather than to human albumin (Hanada et al. 2000). Hage and Sengupta (1999) showed that digitoxin and acetyldigitoxin were bound strongly to a single common binding site on human serum albumin such that neither compound showed any competition with warfarin or L-tryptophan, indicating the presence of a separate binding site on HSA. Wang et al. (1998) reported that fenopropfen was found to bind to two classes of sites on human serum albumin. They also found that ibuprofen and palmitic acid significantly decreased the extent of binding of fenopropfen to HSA.

In summary, this study showed that PB significantly bound to HSA, while DEET and permethrin, did not significantly bind in vitro to HSA. The absence of interaction of both compounds at a binding site shows that their interaction with serum albumin is not a major factor in the enhanced toxicity following combined administration in vivo.

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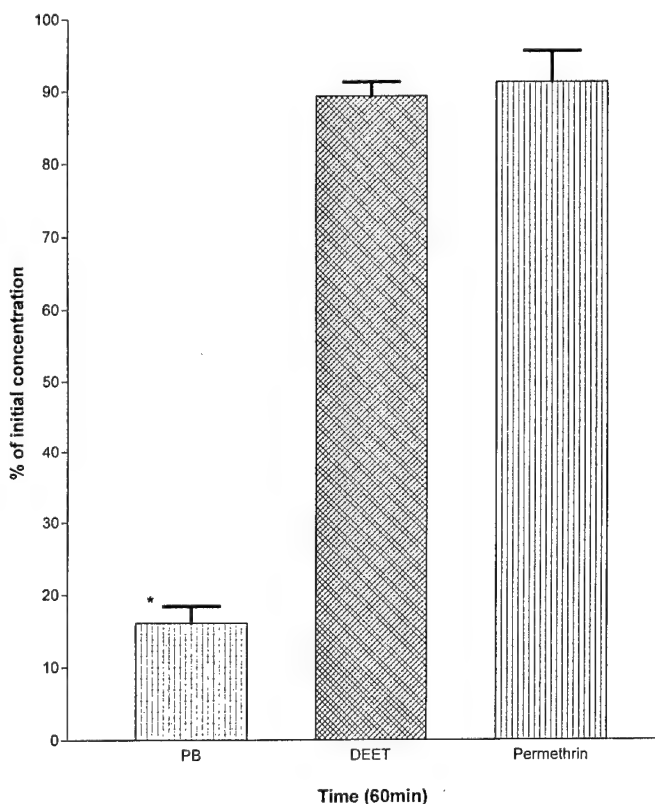


Fig. 5. Levels of pyridostigmine bromide (PB), DEET (*N,N*-diethyl-*m*-toluamide) and permethrin following incubation of a combination of the three compounds with human serum albumin. The results are expressed as mean \pm SD of ten samples from five experiments. * $P < 0.01$; the difference is considered statistically significant if $P < 0.05$

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Disruption of the Blood–Brain Barrier and Neuronal Cell Death in Cingulate Cortex, Dentate Gyrus, Thalamus, and Hypothalamus in a Rat Model of Gulf-War Syndrome

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We investigated the effects of a combined exposure to restraint stress and low doses of chemicals pyridostigmine bromide (PB), *N, N*-diethyl-*m*-toluamide (DEET), and permethrin in adult male rats, a model of Gulf-War syndrome. Animals were exposed daily to one of the following for 28 days: (i) a combination of stress and chemicals (PB, 1.3 mg/kg/day; DEET, 40 mg/kg/day; and permethrin, 0.13 mg/kg/day); (ii) stress and vehicle; (iii) chemicals alone; and (iv) vehicle alone. All animals were evaluated for: (i) the disruption of the blood–brain barrier (BBB) using intravenous horseradish peroxidase (HRP) injections and endothelial barrier antigen (EBA) immunostaining; (ii) neuronal cell death using H&E staining, silver staining, and glial fibrillary acidic protein (GFAP) immunostaining; and (iii) acetylcholinesterase (AChE) activity and m2-muscarinic acetylcholine receptors (m2-AChR). Animals subjected to stress and chemicals exhibited both disruption of the BBB and neuronal cell death in the cingulate cortex, the dentate gyrus, the thalamus, and the hypothalamus. Other regions of the brain, although they demonstrated some neuronal cell death, did not exhibit disruption of the BBB. The neuropathological changes in the above four brain regions were highly conspicuous and revealed by a large number of HRP-positive neurons (21–40% of total neurons), a decreased EBA immunostaining (42–51% reduction), a decreased number of surviving neurons (27–40% reduction), the presence of dying neurons (4–10% of total neurons), and an increased GFAP immunostaining (45–51% increase). These changes were also associated with decreased forebrain AChE activity and m2-AChR (19–25% reduction). In contrast, in animals exposed to stress and vehicle or chemicals alone, the above indices were mostly comparable to that of animals exposed to vehicle alone. Thus, a combined exposure to stress and low doses of PB, DEET, and permethrin leads to significant brain injury. The various neurological symptoms reported by Gulf-War veterans could be linked to this kind of brain injury incurred during the war. © 2002 Elsevier Science (USA)

Key Words: brain injury; Gulf-War syndrome; glial hypertrophy; glial fibrillary acidic protein; neuron degeneration.

INTRODUCTION

Many Persian Gulf War (PGW) veterans have complained of illnesses affecting the nervous and muscu-

loskeletal systems (Amato *et al.*, 1997; Haley *et al.*, 2000a,b). These include chronic fatigue, muscle pain, brain abnormalities, ataxia, inability to concentrate, forgetfulness, and behavioral symptoms (Institute of Medicine, 1995; Haley *et al.*, 2000a,b). During the PGW, the veterans were exposed to a combination of biological, psychological, chemical, and stressful envi-

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ronments. To begin with, to protect against the potential exposure to biological and chemical weapons, the United States and other troops received anthrax vaccine, botulinum toxin vaccine, and 21 tablets of pyridostigmine bromide (PB). The drug PB was employed as a prophylactic treatment to protect against organophosphate nerve agents (Persian Gulf Veterans Coordinating Board, 1995). Second, chemical sensors and alarms employed throughout the region as warning devices to chemical and biological attacks were psychologically challenging, as they were extremely sensitive and could be triggered by organic solvents, vehicle exhaust fumes, insecticides, and chemical warfare agents. Third, pesticides were widely used by troops to combat the ubiquitous insect and rodent populations in the region. The pesticides included organophosphate chemicals, the insect repellent *N,N*-diethyl-*m*-toluamide (DEET), and the insecticide permethrin (Institute of Medicine, 1995; Haley & Kurt, 1997; Haley *et al.*, 1997; van Haaren *et al.*, 2001; Institute of Medicine, 1995). In light of the above pattern of exposure experienced by the troops during the PGW, it is generally believed that the neurological symptoms displayed by PGW veterans are due to a synergistic interaction of PB with other chemicals such as DEET and permethrin and the stress (Institute of Medicine, 1995; Friedman *et al.*, 1996; Hyams *et al.*, 1996; David *et al.*, 1997; Everson *et al.*, 1999).

The drug PB is a reversible cholinesterase inhibitor commonly used for the treatment of myasthenia and as prophylactic protection against organophosphate nerve agents (Sapolsky, 1996; Li *et al.*, 2000). PB is a quaternary molecule and it is believed that, under normal healthy conditions, it does not cross the blood-brain barrier (BBB) (Birtley *et al.*, 1966). PB is known to shield acetylcholinesterase (AChE) from nerve agent poisoning by reversible inhibition of 30–40% of the AChE in the peripheral nervous system (Blick *et al.*, 1991; Wolthius & van Wersch, 1984; Wolthius *et al.*, 1995). However, PB overdose affects both the central and the peripheral nervous systems (Abou-Donia *et al.*, 1996; Glass-Marmor *et al.*, 1996; Wilson *et al.*, 1998; Li *et al.*, 2000). For example, an overdose of PB can cause apoptotic cell death in selected brain regions (Li *et al.*, 2001) and mitochondrial damage in pre- and postsynaptic regions of the neuromuscular junction (Bowman *et al.*, 1989; Drake-Baumann & Seil, 1999).

The chemical DEET is the most commonly used insect repellent (Veltri *et al.*, 1994). The precise target of DEET is currently unknown (Chaney *et al.*, 2000; Robbins & Cherniack, 1986; McConnell *et al.*, 1986). However, extensive and repeated topical application

of DEET leads to human poisoning including death (Gryboski *et al.*, 1961; Roland *et al.*, 1985; Edwards & Johnson, 1987). The symptoms associated with DEET poisoning are characterized by tremors, restlessness, difficulty in speech, seizures, impairment of cognitive function, and coma (McConnell *et al.*, 1986). Extremely high levels of DEET exposure have been reported to cause spongiform myelinopathy (Verschoyle *et al.*, 1992). Permethrin, a synthetic pyrethroid, is a widely used insecticide (Casida *et al.*, 1983; Vijverberg & van den Bercken, 1990). Its insecticidal activity persists for several weeks following a single application. Permethrin intoxication results as a consequence of the sustained opening of sodium channels leading to repetitive discharges after a single stimulus (Narahashi, 1985). This repetitive nerve action is associated with tremor, hyperactivity, ataxia, convulsions, and, in some cases, paralysis.

Stress is a common experience of daily life and all organisms have evolved mechanisms and strategies to deal with crucial alterations in their internal and external environment. A limited stress can actually exert beneficial effects on the brain function, particularly the promotion of plasticity and the enhancement of learning and memory performance (Willner, 1991; Zilles, 1992; Stout & Nemoroff, 1994; Magarinos & McEwen, 1995; Henry & Stephens, 1977; Fuchs & Flugge, 1998). However, a persistent and higher degree of stress can cause deleterious effects on the brain including the disruption of the BBB (Bradbury, 1979; Barryd *et al.*, 1985; Bryan, 1990; Sharma *et al.*, 1991; Fuchs & Flugge, 1998). The potential mechanisms by which stress alters the BBB include: (i) activation of vasoactive mediators such as histamine released from perivascular brain mast cells (Zhuang *et al.*, 1990; Sant & Theoharides, 1994; Theoharides, 1994; Esposito *et al.*, 2001); (ii) activation of the hypothalamic-pituitary-adrenal (HPA) axis through the release of corticotrophin-releasing hormone (CRH) resulting in the secretion of catecholamines and glucocorticoids leading to hippocampal neuronal injury (De Souza, 1987; Sapolsky, 1992; Chrousos, 1995; Baram & Ribak, 1996; Baram *et al.*, 1997); and (iii) interaction of catecholamine and glucocorticoid effects with glutamatergic neurotransmission (Gilad *et al.*, 1990; Sapolsky, 1996). Thus, exposure to drugs and chemicals under stressful conditions is highly detrimental to the CNS. However, several factors play a role in the effects of stress on BBB, particularly the type and the intensity of stress (such as social, physical exercise, swimming, and restraint stress) and the age and sex of animals (Ohno *et al.*, 1989; Aloisi *et al.*, 1997; Friedman *et al.*, 1996; Telang *et*

al., 1999; Grauer *et al.*, 2000). For example, PB treatment with swim stress allows PB to enter the brain and causes a significant inhibition of brain AChE (Keele *et al.*, 1991; Friedman *et al.*, 1996); however, a combined exposure to PB with many other types of relatively milder stress causes no change in brain AChE activity (Lallement *et al.*, 1998; Telang *et al.*, 1999; Grauer *et al.*, 2000; Ovadia *et al.*, 2001). Thus, PB treatment in the presence of a moderate level of stress is not detrimental to the CNS.

However, the development of Gulf-War syndrome is likely due to a combined exposure to multiple factors such as the chemicals DEET, permethrin, and PB and stress. Our earlier studies demonstrate that subchronic exposure to DEET and permethrin causes significant pathological changes in the brain, and a concurrent exposure to larger doses of PB, DEET, and permethrin results in increased neurotoxicity (Abou-Donia *et al.*, 1996; Abdel-Rahman *et al.*, 2001). However, the effects of a combined exposure to moderate levels of stress and the chemicals PB, DEET, and permethrin are unknown. Because of the relevancy of the latter model to the Gulf-War syndrome, we investigated the effects of daily exposure to moderate levels of stress (i.e., restraint stress) and low doses of PB, DEET, and permethrin for 28 days on the permeability of the BBB and neuronal survival in different brain regions. The exposure paradigm employed closely simulated the daily exposure experienced by veterans during the PGW.

MATERIALS AND METHODS

Chemicals

Technical-grade (94%) permethrin (\pm -*cis/trans*-, 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid (3-phenoxyphenyl) methyl ester was obtained from Roussel Uclaf Corporation (Pasadena, TX). The chemicals DEET ($\geq 97\%$ *N*, *N*-diethyl-*m*-toluamide), pyridostigmine bromide ($\geq 99\%$, 3-dimethylaminocarbonyloxy *N*-methylpyridinium bromide), acetylthiocholine iodide, butyrylthiocholine iodide, horseradish peroxidase (HRP) type VI, and diaminobenzidine tetrahydrochloride were purchased from Sigma (St. Louis, MO). The chemical [^3H]AF-DX384 (sp. activity 106.5 Ci/mmol) was from New England Nuclear (Boston, MA). All other chemicals and reagents were of the highest purity available from commercial sources.

Animals and Treatment Protocol

Male Sprague-Dawley rats weighing 225–250 g were obtained from Zivic-Miller Laboratories (Allison Park, PA). The animals were randomly assigned to control and treatment groups and housed at 21–23°C with a 12-h light/dark cycle. They were supplied with Purina Certified Rodent Chow (Ralston Purina Co., St. Louis, MO) and tap water ad libitum. The rats were allowed to adjust to their environment for a week before treatment. Animal care was in accordance with U.S. Army guidelines and approved by the Duke University Animal Care and Use Committee. Animals in Group 1 ($n = 20$) were treated daily with PB (1.3 mg/kg/day, oral in water), DEET (40 mg/kg/day, dermal in 70% ethanol), and permethrin (0.13 mg/kg/day, dermal in 70% ethanol) for 28 days. Animals in Group 2 ($n = 20$) were treated daily with all of the above chemicals and also subjected to 5 min of restraint stress every day for the duration of the experiment. This was done after the chemical treatment by placing the rats in a Plexiglas cylinder. Animals in Group 3 ($n = 20$) were treated with a dermal application of 70% ethanol and oral water (1 ml/kg) and subjected to the restraint stress as described above. Animals in Group 4 ($n = 20$) were treated with a dermal application of 70% ethanol and oral water daily for 28 days. We selected 5 min of restraint stress per day in these experiments based on our pilot experiments on the effect of different durations of restraint stress (5, 10, 15, 20, and 60 min) and PB exposure on cortisol levels. In the latter time-course study, we found that a significant increase in cortisol occurs with at least 15–20 min of restraint stress per day. However, we selected 5 min of restraint stress per day in the present study, as we wanted to study the effect of moderate stress when combined with chemicals PB, DEET, and permethrin on BBB permeability and neuronal survival.

Histopathological Analyses

Following the exposure regimen described above, five animals from each group were anesthetized with pentobarbital (100 mg/kg) and perfused through the heart with saline followed by 4% paraformaldehyde and 0.1% glutaraldehyde in Tris buffer. The brains and livers were removed, postfixed, and embedded in paraffin according to standard histological techniques. Six-micrometer-thick coronal sections were cut through the different regions of the forebrain. In every brain, representative sections through the cerebral cortex, hippocampus, thal-

amus, and hypothalamus were processed and stained with hematoxylin and eosin (H&E) for light microscopic observation. A few sections through the liver were also processed for similar H&E staining.

Analysis of Blood-Brain Barrier Permeability Using Horseradish Peroxidase

Five animals in each group were injected with HRP type VI (10% solution in saline) at a dose of 135 mg/kg (iv) to give a final concentration of 30–35 mg/animal. Thirty minutes after the injection of HRP, animals were perfused with a fixative containing 4% paraformaldehyde and 2% glutaraldehyde in phosphate-buffered saline (PBS). Brains were removed, postfixed, and embedded in paraffin, and sections (8 micron thick) were cut using a microtome. Representative sections (approximately corresponding to every 30th section) through the forebrain were deparaffinized and processed for HRP immunohistochemistry. For this, sections were blocked with 10% goat serum in 0.1 M PBS for 20 min and incubated with a primary antibody against HRP (Sigma) for 45 min at 40°C. Sections were then incubated with an appropriate secondary antibody solution in 0.1 M PBS for 20 min. Sections were developed by the ABC method of Hsu *et al.*, (1981) using reagents from Vector Labs (Burlingame, CA). Sections were examined using a Nikon E600 microscope.

Glial Fibrillary Acidic Protein and Endothelial Barrier Antigen Immunohistochemistry and Silver Staining

The sections through the forebrain were immunohistochemically stained using antibodies for the glial fibrillary acidic protein (GFAP, 1:10,000; Dako) and the endothelial barrier antigen (EBA, Sternberger Monoclonals, 1:1000 dilution; Rosenstein *et al.*, 1992; Jensen *et al.*, 1998) with the ABC method (Hsu *et al.*, 1981), using reagents from Vector Labs. To unequivocally detect the presence of neuronal degeneration, silver staining was performed on additional sections through the forebrain. The processing of sections with FD Neurosilver kit (FD NeuroTechnologies, Ellicott City, MD) stains only the degenerating neuronal elements (soma as well as dendrites) in paraformaldehyde-fixed brain tissues (Betarbet *et al.*, 2000). The processing of sections for silver staining was done according to the manufacturer's protocol. Sections were examined using bright-field optics in a Nikon E600 microscope.

Quantitative Evaluation of the Number of Surviving and Dying Neurons and HRP-Positive Neurons in Different Brain Regions

The numerical density of surviving and dying neurons in H&E-stained sections and HRP-positive neurons in HRP-immunostained sections was measured per square millimeter of the tissue for the cingulate cortex, the granule cell layer of the dentate gyrus, the lateral dorsal nucleus of the thalamus, and the dorsomedial nucleus of the hypothalamus. These regions were specifically selected for this analysis because the experiments on BBB permeability using HRP injections and EBA immunostaining suggested a significant leakage of the BBB in these brain regions in animals exposed to both stress and chemicals. The leakage of BBB in only certain regions of the brain observed in this study is consistent with earlier reports on regional differences in BBB (Belova & Jonsson, 1982). It is likely that BBB leakage in only certain areas of the brain following combined exposure to stress and chemicals reflects a relative weakness of the vessel walls in these regions compared to other regions that remain intact.

Five sections through each of the above brain regions were employed for these measurements in every animal belonging to the following four groups: (a) control animals ($n = 5$); (b) animals treated with stress alone ($n = 5$); (c) animals treated with chemicals alone ($n = 5$); and (d) animals treated with both stress and chemicals ($n = 5$). Measurements in sections from various groups were performed in a blinded fashion using experimental codes. The coding was such that animal treatments were not known during the measurement; however, sections that came from the same animal were identified. All measurements were performed using a Nikon E600 microscope equipped with an eyepiece grid. At a magnification of 400 \times (using 40X objective lens and 10X eyepieces), both surviving and dying neurons within two unit areas in each section were measured. The unit area selected for measurements varied for different regions of the brain, depending on the availability of the overall area for different layers.

The area measured was 0.063 mm² for the cingulate cortex (layers II and III), the lateral dorsal nucleus of the thalamus, and the dorsomedial nucleus of the hypothalamus and 0.013 mm² for the dentate granule cell layer. For the measurement of the number of surviving neurons, only those neurons that exhibited hematoxylin-stained nucleus with a clear nucleolus were counted. For measurement of the dying neurons,

only those neurons that exhibited dense eosinophilic staining in both soma and proximal dendrites were counted. Finally, the density of neurons per unit area was transformed to the numerical density per square millimeter of respective brain region. For measurement of HRP-positive neurons, both total neurons and neurons that exhibited dense HRP labeling within the cytoplasm of both soma and apical dendrites were counted. The percentage of HRP-positive neurons among the total neurons was then calculated for every region. The mean value for each brain region was calculated separately for every animal by using data from five sections (two areas/section) before the means and standard errors were determined for the total number of animals included per group. Mean values between different groups of animals were compared separately for each of the above brain regions using one-way ANOVA with the Student-Newman-Keuls multiple comparison post hoc test.

Morphometric Analyses of GFAP- and EBA-Immunopositive Elements in Different Brain Regions

Morphometric analyses of GFAP-positive and EBA-positive immunoreactive structures in different regions were performed using Scion Image for Windows, which is based on the NIH Image software for the Macintosh (Scion Corp., Frederick, MD). For every brain region, two sections were measured in each animal. All data were collected blind to the experimental codes and means were calculated for each animal individually before the means were determined for the five animals per group. Statistical comparisons on GFAP and EBA measurements in different brain regions between control and treated groups utilized ANOVA with the Student-Newman-Keuls multiple comparisons post hoc test.

The areas occupied by the GFAP- or the EBA-positive immunoreactive structures per unit area of tissue (0.0176 mm^2 in area) were determined for the cingulate cortex, the dentate gyrus, the lateral dorsal nucleus of the thalamus, and the dorsomedial nucleus of the hypothalamus. For every region, the microscopic image (using 10X objective lens) was transferred to a computer screen by focusing the appropriate area of the immunostained section with a Nikon E600 microscope equipped with a digital camera (DAGE-MTI, CCD100) connected to an IBM computer. The same intensity of light in the microscope and the same parameters in the digital camera were used to digitize all samples from different animals. Images in Scion Im-

age are two-dimensional arrays of pixels (picture elements). Pixels are represented by 8-bit unsigned integers, ranging in value from 0 to 255. Scion Image displays zero pixels as white and those with a value of 255 as black. The background and target values were set to 145 and 255, respectively, following digitization of the original gray value image on the computer screen. These values were determined by selecting the background and target areas in several sections from both control and treated animals before commencing the measurements on coded slides for statistical analysis. This scale eliminated most of the background staining and retained all target (GFAP or EBA immunopositive) structures in the range (145–255). The binary image of the GFAP- or EBA-immunopositive elements was then generated by selecting a suitable threshold value (which varied from 155 to 165) to include all GFAP- or SMI-71-positive structures without any background. The final binary image was crosschecked with the original gray value image by alternating the two images on the computer screen.

Finally, the image was frozen and the area occupied by the GFAP- or SMI-71-positive structures in the field was measured by selecting the "Analyze particles" command of the Scion Image program. This way, the area of individual particles (i.e., the GFAP- or the EBA-immunoreactive structures) in the selected field was measured, and the sum area of all particles was stored for further calculations and statistical analyses. Since spatial calibration of the image was performed in micrometers using the "Set Scale" function of the program prior to measurements, the results from area measurements were obtained in square micrometers and converted later into square millimeters. The mean values for each of the four brain regions were calculated separately for each animal by using data from five sections before the means and standard errors were determined for the total number of animals ($n = 5$) included per group. Mean values between different groups of animals were compared separately for each of the above brain regions using one-way ANOVA with the Student-Newman-Keuls multiple comparison post hoc test.

Acetylcholinesterase, Butyrylcholinesterase Activities, and m2-Muscarinic Acetylcholine Receptor (m2-AChR) Binding Assays

The acetylcholinesterase activity in the forebrain and butyrylcholinesterase (BChE) activity in the plasma was assayed according to the method de-

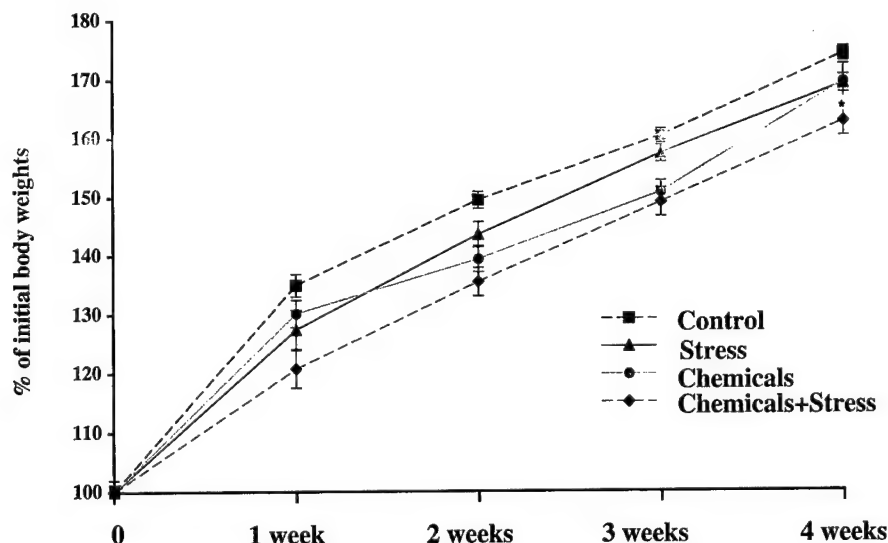


FIG. 1. Effect of daily administration of pyridostigmine bromide (PB), DEET, and permethrin, with or without stress, on the body weight of rats. Data are presented as percentages of the initial body weight. The percentage of initial body weight for controls (mean \pm SEM) is 134.93 ± 1.895 (Week 1), 149.43 ± 1.428 (Week 2), 160.17 ± 1.157 (Week 3), and 174.09 ± 1.215 (Week 4). Analysis with one-way ANOVA revealed significant differences between groups ($P < 0.001$). The post hoc analysis showed that animals treated with both stress and chemicals exhibited a significant decrease in body weight, compared to both control animals ($P < 0.01$) and animals treated with stress alone ($P < 0.05$).

scribed in earlier studies (Ellman *et al.*, 1961) but modified for assay in a Molecular Devices VERSA Max Tunable Microplate Reader (Molecular Devices Corp., Sunnyvale, CA), as previously described (Abou-Donia *et al.*, 1996). Protein concentration was determined by the method of Smith *et al.* (1985). For the assay of m2-muscarinic acetylcholine receptor (m2-AChR), the forebrain tissues were homogenized in 10 mM phosphate buffer, pH 7.4, and centrifuged at 40,000g for 10 min and the membranes were suspended in the same buffer at a protein concentration of 1.5–2.5 mg/ml, as described by Huff *et al.* (1994). The m2-AChR binding was carried out using m2-selective ligand [3 H]AFDX 384 at room temperature for 60 min, as described by Slotkin *et al.* (1999).

RESULTS

General Observations

The clinical condition of animals did not show any apparent difference among different treatment groups or between the treatment groups and the control group. Figure 1 illustrates body weight data for animals in different groups. The animals treated with both stress and chemicals PB, DEET, and permethrin showed a significant weight loss compared to the

vehicle-treated control animals between weeks 2 and 4 (8 to 14% decrease, $P < 0.01$) and animals treated with stress alone between weeks 1 and 2 ($P < 0.05$).

Alterations in BBB Permeability Using HRP Injections

Evaluation of the distribution of HRP within the parenchyma of the brain using HRP immunohistochemistry demonstrated a large number of HRP-positive neurons within four regions of the brain in animals treated with both stress and chemicals. These include the cingulate cortex, the dentate gyrus of the hippocampal formation, the thalamus, and the hypothalamus (Figs. 2, 3, 4, and 5). In the cingulate cortex, a large number of HRP-positive neurons were observed in the outer granule cell layer (layer II) and pyramidal cell layers (layers III and V). In many pyramidal neurons of the layers III and V, a large amount of HRP was evident within the cytoplasm of both soma and apical dendrites (Fig. 2). Sections through the anterior and posterior regions of the cingulate cortex revealed similar staining of the neurons. In the dentate gyrus, a large number of dentate granule cells were positive for HRP in the medial part of the dentate gyrus comprising the crest and portions of the upper and lower blades (Fig. 3). A few neurons in

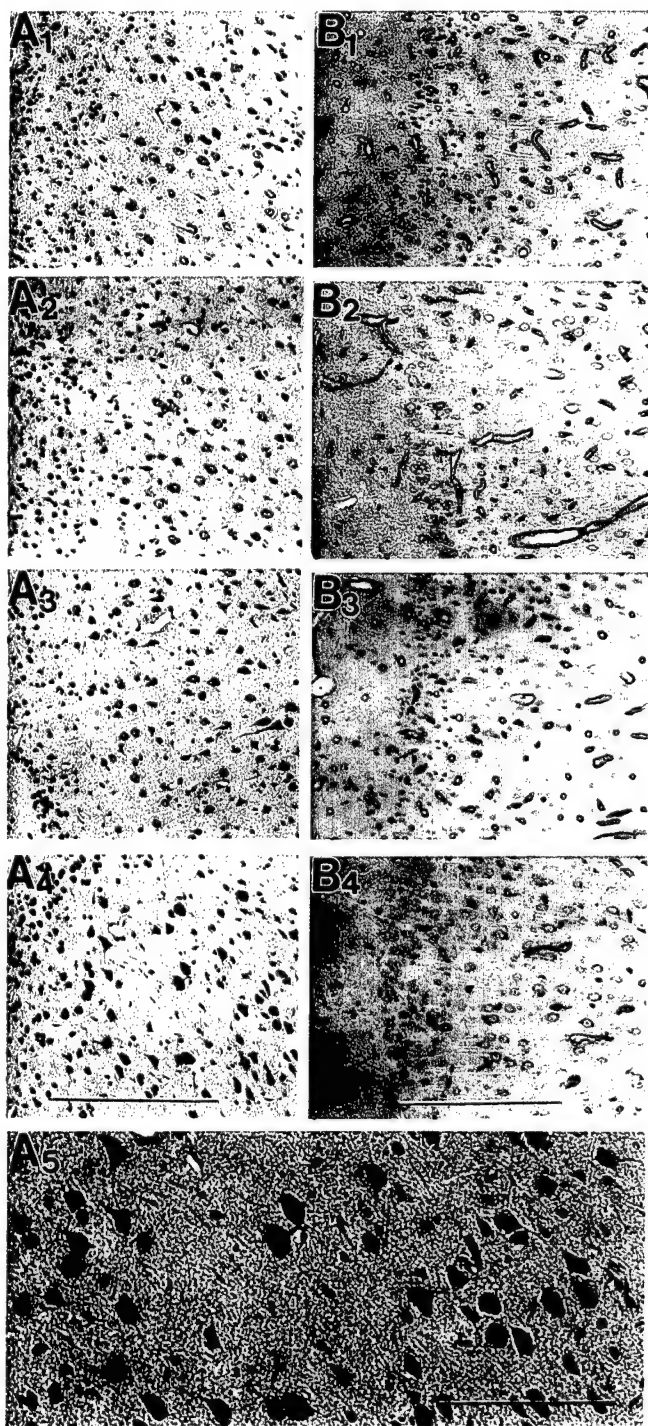


FIG. 2. Horseradish peroxidase (HRP; A1–A5) and endothelial barrier antigen (EBA; B1–B4) immunostaining of the cingulate cortex from different groups. A1 and B1 are from the vehicle-treated control group. A2 and B2 are from the group treated with stress alone. A3 and B3 are from the group treated with chemicals alone. A4 and B4 are from group treated with both stress and chemicals. A5 shows a magnified view of a region from A4. The neurons positive for HRP (in layers II and III) are observed only in the group

the dentate hilus were also positive for HRP. Evaluation of the sections through the septal and temporal levels of the hippocampal formation revealed that the BBB was leaky throughout the septotemporal axis of the dentate gyrus. In the thalamus, a large number of HRP-positive neurons were observed mainly in the lateral dorsal nucleus (Fig. 4). The HRP-positive neurons in this nucleus were either triangular or multipolar. In the hypothalamus, many hypothalamic nuclei demonstrated HRP-positive neurons but the highest number appeared in the dorsomedial nucleus (Fig. 5). In contrast, control animals and animals subjected to stress and chemicals alone exhibited only a few HRP-positive neurons in the above regions (Figs. 2, 3, 4, and 5). Further, the amount of HRP within HRP-positive neurons in the latter groups appeared very small. The absence of densely stained HRP-positive neurons within the above brain regions in the control group further suggests that the above brain regions do not have significantly leaky BBB under normal conditions. Thus, the presence of a large number of very densely stained HRP-positive neurons in the cingulate cortex, the dentate gyrus of the hippocampal formation, the lateral dorsal nucleus of the thalamus, and the dorsomedial nucleus of the hypothalamus in only the animals treated with both stress and chemicals suggests that these brain regions represent areas of the brain where severe BBB damage occurs after a combined exposure to stress and the chemicals PB, DEET, and permethrin.

Extent of HRP-Positive Neurons in Different Regions of the Brain

The measurement of the HRP-positive neurons per square millimeter of tissue in different brain regions revealed a significant number of HRP-positive neurons in animals treated with both stress and chemicals, in comparison to animals treated with stress, chemicals, or vehicle alone (Fig. 6). In animals treated with stress and chemicals, the percentage of HRP-positive neurons among total neurons was significantly greater

that received both stress and chemicals treatment (A4). In these neurons HRP is localized in the cytoplasm and proximal dendrites (A5). Further, the density of EBA-positive elements is significantly reduced in the group that received both stress and chemicals treatment (B4), in comparison to other groups. Note that both parameters in animals treated with chemicals (A2, B2) or stress alone (A3, B3) appear comparable to the vehicle-treated control group (A1, B1). Scale bar, A1–A4 and B1–B4 = 175 μ m; A5 = 50 μ m.

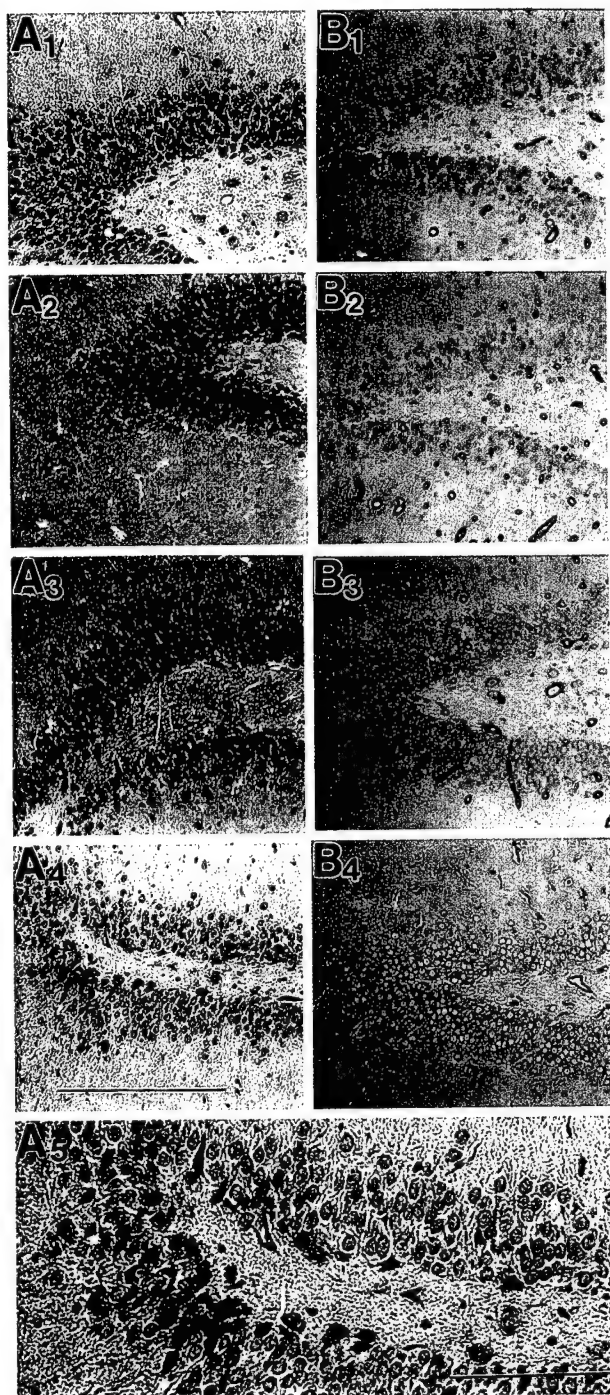


FIG. 3. Horseradish peroxidase (HRP; A1–A5) and endothelial barrier antigen (EBA; B1–B4) immunostaining of the dentate gyrus from different groups. A1 and B1 are from the vehicle-treated control group. A2 and B2 are from the group treated with stress alone. A3 and B3 are from the group treated with chemicals alone. A4 and B4 are from group treated with both stress and chemicals. A5 shows a magnified view of a region from A4. The neurons positive for HRP (in the dentate granule cell layer and the dentate hilus) are observed only in the group that received both stress and

chemicals treatment with stress, chemicals, or vehicle alone. The percentage of HRP-positive neurons was 28% in the cingulate cortex ($P < 0.001$), 21% in the dentate granule cell layer ($P < 0.001$), 33% in the lateral dorsal nucleus of the thalamus ($P < 0.001$), and 40% in the dorsomedial nucleus of the hypothalamus ($P < 0.05$). The percentage of HRP-positive neurons among total neurons in animals treated with stress or chemicals alone was minimal and comparable to the percentage in vehicle-treated control animals in all of the above brain regions except the thalamus (Fig. 6). In the thalamus, the stress alone group exhibited a significant percentage of HRP-positive neurons, compared to animals treated with chemicals or vehicle alone ($P < 0.01$). Thus, quantitative analyses of the percentage of HRP-positive neurons among total neurons demonstrated a synergistic effect following exposure to both chemicals and stress.

Alterations in the BBB Using EBA Immunostaining

The alterations in BBB in different animal groups were also assessed by EBA immunostaining which shows BBB protein in brain capillaries and also in smaller vessels invading the brain parenchyma. Earlier studies have demonstrated that a dramatic reduction in EBA staining is indicative of an alteration in the BBB (Jensen *et al.*, 1998). We found that animals treated with both chemicals and stress exhibited dramatic reductions in EBA-immunoreactive elements in all of the brain regions where HRP-positive neurons were found (i.e., the cingulate cortex, the dentate gyrus, the lateral dorsal nucleus of the thalamus, and the dorsomedial nucleus of the hypothalamus; Figs. 2, 3, 4, and 5). Other regions of the brain in animals treated with both chemicals and stress also appeared to have some decrease in EBA staining, in comparison to the vehicle-treated control animals (data not shown). In animals treated with either stress or chemicals alone, the extent of EBA-positive structures appeared closer to that of the vehicle-treated control animals.

chemicals treatment (A4). In these neurons HRP is localized in the cytoplasm and proximal dendrites (A5). Further, the density of EBA-positive elements is significantly reduced in the group that received both stress and chemicals treatment (B4), in comparison to other groups. Note that both parameters in animals treated with chemicals (A2, B2) or stress alone (A3, B3) appear comparable to that of the vehicle-treated control group (A1, B1). Scale bar, A1–A4 and B1–B4 = 175 μm ; A5 = 50 μm .

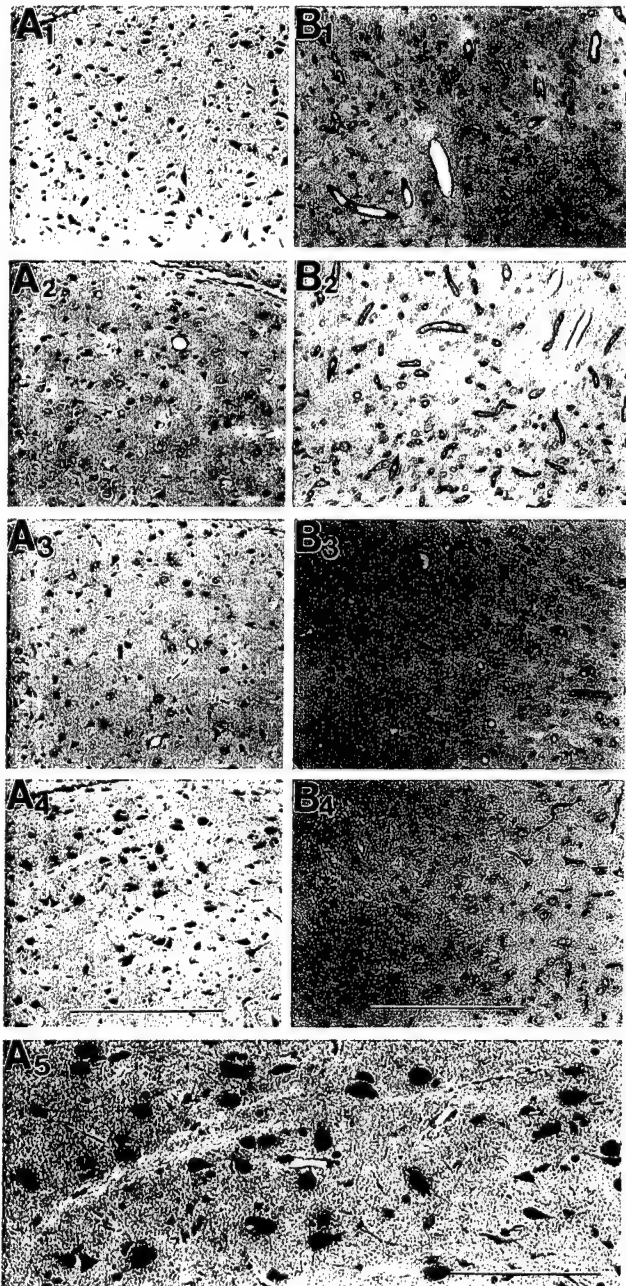


FIG. 4. Horseradish peroxidase (HRP; A1–A5) and endothelial barrier antigen (EBA; B1–B4) immunostaining of the lateral dorsal nucleus of the thalamus from different groups. A1 and B1 are from the vehicle-treated control group. A2 and B2 are from the group treated with stress alone. A3 and B3 are from the group treated with chemicals alone. A4 and B4 are from group treated with both stress and chemicals. A5 shows a magnified view of a region from A4. The neurons positive for HRP are observed only in the group that received both stress and chemicals treatment (A4). In these neurons HRP is localized in the cytoplasm and proximal dendrites (A5). Further, the density of EBA-positive elements is significantly reduced in the group that received both stress and chemicals treatment (B4), in comparison to other groups. Note that both paramet-

Quantification of the area of EBA-immunoreactive elements per unit area of the cingulate cortex, the dentate gyrus, the lateral dorsal nucleus of the thalamus, and the dorsomedial nucleus of the hypothalamus demonstrated the following. Animals treated with both stress and chemicals showed a dramatic decrease in EBA immunostaining in all of the above brain regions, in comparison to the vehicle-treated control animals (Fig. 7). The overall reduction in the EBA-immunoreactive structures was 42% in the cingulate cortex ($P < 0.01$), 48% in the dentate gyrus ($P < 0.001$), 51% in the lateral dorsal nucleus of the thalamus ($P < 0.05$), and 43% in the dorsomedial nucleus of the thalamus. Animals treated with stress or chemicals alone showed no significant reductions in any of the above brain regions (Fig. 7). Thus, the findings on the disruption of the BBB analyzed with HRP penetration and EBA immunostaining are complementary and together suggest a significant disruption of the BBB in animals exposed to both stress and the chemicals PB, DEET, and permethrin.

Histopathological Changes in the Brain

Evaluation of the brain sections stained with H&E clearly revealed neuronal degeneration in rats treated with both chemicals and stress but not in animals treated with chemicals, stress, or vehicle alone. Degrading (or dying) neurons were characterized by eosinophilic staining of both cell body and proximal dendrites. In contrast, the healthy neurons in the same section exhibited a hematoxylin-stained nucleus (with a clear nucleolus) within a lightly eosin-stained perinuclear cytoplasm. The dying neurons were seen in multiple brain regions in animals treated with both stress and chemicals. However, our detailed analysis focused on the cingulate cortex, the dentate gyrus, the lateral dorsal nucleus of the thalamus, and the ventromedial nucleus of the hypothalamus, as these regions represent areas of the brain where a significant increase in BBB permeability occurs after exposure to both stress and chemicals.

In the cingulate cortex, the degenerating neurons were conspicuous in layers II, III, and V. The majority of degenerating neurons in layers III and V were of pyramidal type with prominent eosinophilic apical

ters in animals treated with chemicals (A2, B2) or stress alone (A3, B3) appear comparable to that of the vehicle-treated control group (A1, B1). Scale bar, A1–A4 and B1–B4 = 175 μm ; A5 = 50 μm .

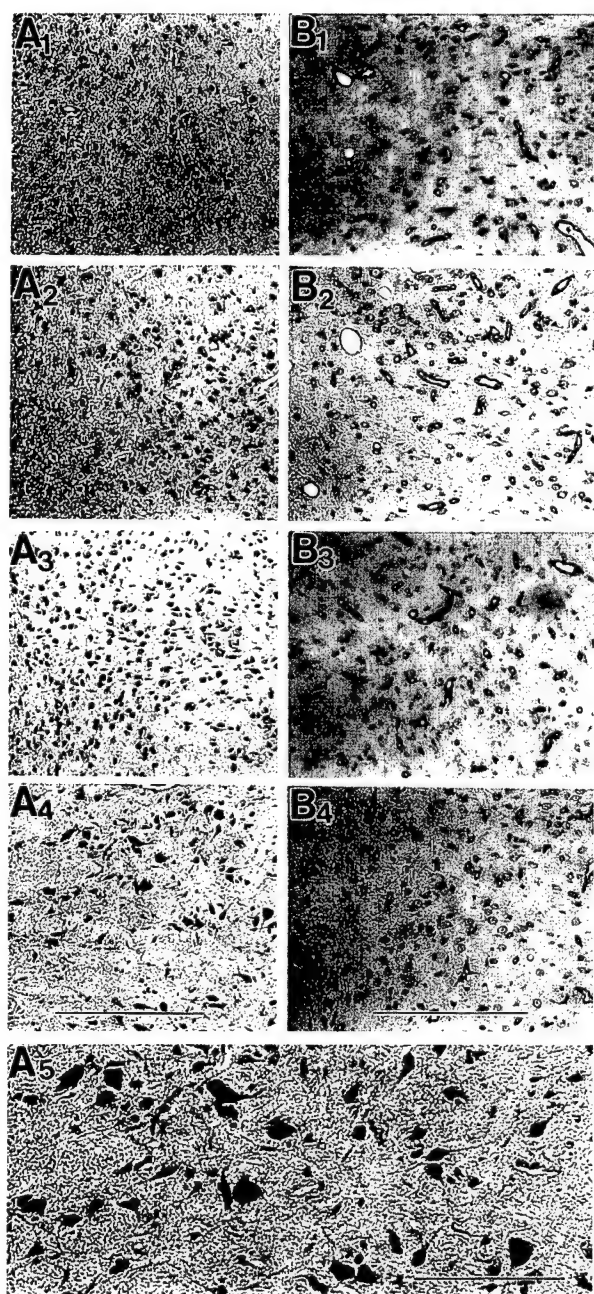


FIG. 5. Horseradish peroxidase (HRP; A1–A5) and endothelial barrier antigen (EBA; B1–B4) immunostaining of the dorsomedial nucleus of the hypothalamus from different groups. A1 and B1 are from the vehicle-treated control group. A2 and B2 are from the group treated with stress alone. A3 and B3 are from the group treated with chemicals alone. A4 and B4 are from group treated with both stress and chemicals. A5 shows a magnified view of a region from A4. The neurons positive for HRP are observed only in the group that received both stress and chemicals treatment (A4). In these neurons HRP is localized in the cytoplasm and proximal dendrites (A5). Further, the density of EBA-positive elements is significantly reduced in the group that received both stress and chemicals treatment (B4), in comparison to other groups. Note that

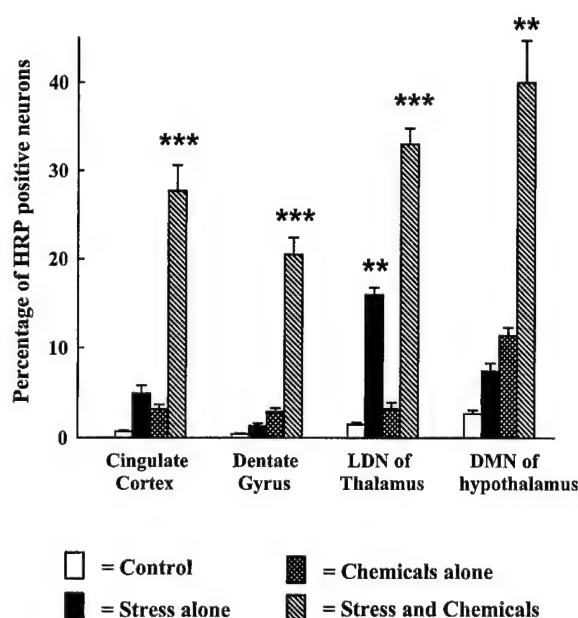


FIG. 6. Histograms show the percentage of HRP-positive neurons in the cingulate cortex, the dentate gyrus, the lateral dorsal nucleus of the thalamus, and the dorsomedial nucleus of the hypothalamus in different groups. Values represent means and standard errors ($n = 5$ per group). Analysis with one-way ANOVA shows a significant increase in HRP-positive neurons in animals treated with stress and chemicals, in comparison to animals treated with stress, chemicals, and vehicle alone ($P < 0.001$). The percentage of HRP-positive neurons among total neurons in animals treated with both stress and chemicals was 28% in the cingulate cortex, 21% in the dentate granule cell layer, 33% in the lateral dorsal nucleus of the thalamus, and 40% in the dorsomedial nucleus of the hypothalamus. Note that, in animals treated with stress or chemicals alone, there is no significant increase in HRP-positive neurons, in comparison to the vehicle-treated control animals. The only exception was the thalamus, where a significant number of HRP-positive neurons were observed in animals treated with stress alone. $**P < 0.01$; $***P < 0.001$.

dendrites (Fig. 8). In the dentate gyrus, a large number of degenerating neurons were conspicuous in the dentate granule cell layer but the dentate hilus also showed a few degenerating neurons (Fig. 9). A closer examination of the granule cell layer revealed a large number of dying cells in the subgranular zone (i.e., the zone of the dentate gyrus containing dentate progenitor/stem cells; Fig. 9). This may suggest that either

both parameters in animals treated with chemicals (A2, B2) or stress alone (A3, B3) appear mostly comparable to that of the vehicle-treated control group (A1, B1). Scale bar, A1–A4 and B1–B4 = 175 μm ; A5 = 50 μm .

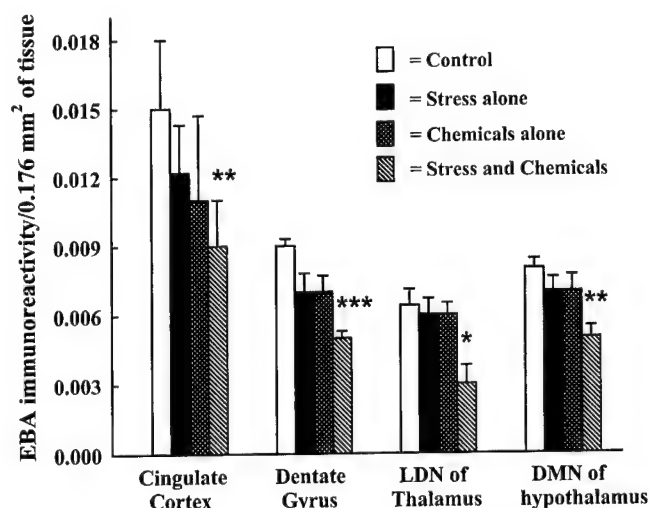


FIG. 7. Histograms show the extent of endothelial barrier antigen (EBA)-immunoreactive elements, in square millimeters per unit (0.176 mm²) of the cingulate cortex, the dentate gyrus, the lateral dorsal nucleus of the thalamus, and the dorsomedial nucleus of the hypothalamus in different groups. Values represent means and standard errors ($n = 5$ per group). Analysis with one-way ANOVA shows a significant decrease in EBA-positive elements in animals treated with stress and chemicals (denoted by asterisks), in comparison to vehicle-treated control animals in all brain regions ($P < 0.001$). The decrease was 42% in the cingulate cortex, 48% in the dentate gyrus, 51% in the lateral dorsal nucleus of the thalamus, and 43% in the dorsomedial hypothalamus nucleus. Note that in animals treated with stress or chemicals alone there is no significant decrease in EBA immunostaining, in comparison to the vehicle-treated control animals. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

granule cell progenitors/stem cells or newly formed granule cells are highly vulnerable to the combined stress and chemicals exposure. In the lateral dorsal nucleus of the thalamus and the dorsomedial nucleus of the hypothalamus, a diffuse neuronal cell death was observed (Figs. 10 and 11). Analyses with silver staining confirmed the presence of dying neurons in all of the above four brain regions (Figs. 8, 9, 10, and 11). The dying neurons exhibited a dense silver staining in both soma and dendrites whereas the healthy or surviving neurons exhibited only a diffuse background staining in the soma, as described earlier (Betarbet *et al.*, 2000).

Analyses of GFAP immunoreactivity also suggested brain injury within the above brain regions in animals treated with both stress and chemicals. The latter animals exhibited an increased number of GFAP-positive astrocytic processes. The astrocytic processes also appeared to be longer and swollen, in comparison to their appearance in the vehicle-treated animals (Figs.

8, 9, 10, and 11). In animals treated with either chemicals or stress alone, the overall GFAP immunoreactivity appeared slightly greater than that of vehicle-treated control animals but not as much as in animals treated with both stress and chemicals. (Figs. 8, 9, 10, and 11). Thus, BBB disruption and neuronal cell death in animals treated with both stress and chemicals within different brain regions were also associated with an increased GFAP immunoreactivity.

Quantification of Surviving and Dying Neurons in Different Regions of the Brain

The measurement of surviving and dying neurons per square millimeter of tissue in different brain regions revealed a significant reduction in the density of surviving neurons and a significant number of dying neurons in animals treated with both stress and chemicals (Fig. 12). However, the density of surviving and dying neurons in animals treated with either stress or chemicals alone was comparable to that of vehicle-treated control animals (Fig. 12). In comparison to vehicle-treated control animals, the density of surviving neurons in animals treated with both stress and chemicals exhibited a 36% decrease in the cingulate cortex ($P < 0.01$), a 40% decrease in the dentate granule cell layer ($P < 0.001$), a 30% decrease in the lateral dorsal nucleus of the thalamus ($P < 0.05$), and a 40% decrease in the dorsomedial nucleus of the hypothalamus ($P < 0.01$). Animals treated with a combination of stress and chemicals also exhibited a significant number of dying neurons in all of the above brain regions, in comparison to animals in other groups ($P < 0.001$; Fig. 12). Thus, quantitative analyses of both surviving and dying neurons clearly demonstrate a significant loss of neurons with exposure to a combination of stress and chemicals but no loss of neurons with exposure to either stress or chemicals alone.

The measurement of GFAP-immunoreactive structures per unit area of tissue demonstrated up-regulation of GFAP immunoreactivity in all of the above brain regions in animals exposed to both stress and chemicals, in comparison to the vehicle-treated control animals (Fig. 13). The increase was 45% in the cingulate cortex ($P < 0.01$), 51% in the dentate gyrus ($P < 0.01$), 48% in the lateral dorsal nucleus of the thalamus ($P < 0.01$), and 50% in the dorsomedial nucleus of the hypothalamus ($P < 0.001$). However, animals treated with either stress or chemicals alone did not exhibit any increase in GFAP immunoreactivity, in comparison to the vehicle-treated control animals ($P > 0.05$; Fig. 13).

Histopathological Changes in the Liver

Examination of sections through the liver stained with H&E in animals treated with both chemicals and stress showed alterations in the hepatic cytoarchitecture, characterized by portal and periportal fibrosis with mononuclear inflammatory cells (data not illustrated). In addition, sinusoidal dilatation and predominant microvacuoles were observed, in comparison to the normal appearance of liver tissue in control animals and animals treated with either stress or chemicals alone. Thus, significant liver damage occurs with combined exposure to stress and chemicals. It is likely that significant liver damage in this group led to an exacerbated effect of chemicals on the brain. This is because significant liver damage can decrease the rate of the detoxification process and lead to an increase in the duration of the availability of chemicals in the body.

Effects on AChE Activity in the Forebrain, BChE Activity in the Plasma, and m2-Muscarinic ACh Receptors Ligand Binding in the Forebrain

AChE activity in the forebrain and plasma BChE activity in different groups at 24 h after the last treatment are illustrated in Fig. 14. Only the animals exposed to both stress and chemicals exhibited a significant decrease in both forebrain AChE activity and plasma BChE activity, in comparison to vehicle-treated control animals and animals treated with either stress or chemicals alone ($P < 0.05$). In view of the liver damage, it is likely that reduced AChE and BChE activities are due to a sustained availability of chemicals in the body as a consequence of decreased rate of detoxification of chemicals. The data for different groups from ligand binding studies for m2-AChR in the forebrain using m2-selective ligand, [3 H]AF-DX-384, are illustrated in Fig. 15. The down-regulation of m2-AChR was clearly evident in animals treated with both stress and chemicals ($P < 0.05$). The animals treated with chemicals alone did not show any change but animals treated with stress alone exhibited a decrease ($P < 0.05$).

DISCUSSION

Our study provides the first evidence for BBB disruption and neuronal cell death in multiple regions of the adult brain following exposure to a combination of stress and low doses of the chemicals PB, DEET, and permethrin. The regions included the cingulate cortex,

the dentate gyrus, the lateral dorsal nucleus of the thalamus, and the dorsomedial nucleus of the hypothalamus. The BBB disruption was evidenced by: (i) perivascular and neuronal accumulation of HRP after intravenous administration of HRP and (ii) dramatic reductions in the immunoreactivity for the BBB protein EBA. The neuronal cell death was evidenced by decreases in the density of surviving neurons and the presence of a large number of dying neurons with H&E and silver staining. These changes were associated with increases in GFAP immunoreactivity and decreases in forebrain AChE activity and m2-muscarinic acetylcholine receptors. In contrast, animals subjected to either stress or chemicals alone did not exhibit the above combination of changes in any of the brain regions.

Collectively, these results underscore that, when combined with stress, exposure to even low doses of PB, DEET, and permethrin can lead to significant brain injury, characterized by an enhanced BBB permeability, diffuse neuronal cell death, an increased GFAP immunoreactivity, and decreased forebrain AChE activity. These findings are of considerable importance as exposure to a combination of stress and the above chemicals is considered a model of the Gulf-War syndrome (Ismail *et al.*, 1999; White *et al.*, 2001). This is because PGW veterans were exposed to all of the above chemicals and the stress related to the combat and/or the posttraumatic stress disorder (Institute of Medicine, 1995). The current findings suggest that the various neurological symptoms observed in PGW veterans (David *et al.*, 1997; Hyams *et al.*, 1996; Everson *et al.*, 1999; Ismail *et al.*, 1999) may be due to a diffuse damage in multiple areas of the brain and likely involved both BBB disruption and neuronal cell death.

Potential Reasons for Alterations in BBB Following Combined Exposure to Stress and Chemicals

The mammalian brain is partially protected from chemical insults by the BBB, which isolates the brain from the plasma and provides the brain with a tightly controlled metabolic environment (Aschner & Gannon, 1994; El-bacha & Minn, 1999; Ovadia *et al.*, 2001). Cerebrovasculature is the site of injury in a number of neurological conditions and injury following exposure to neurotoxins. Damage to the BBB leads to an increased entry of serum proteins and neurotoxins into the brain, changes in the homeostasis and metabolic activities of the brain, and alterations in the vesicular

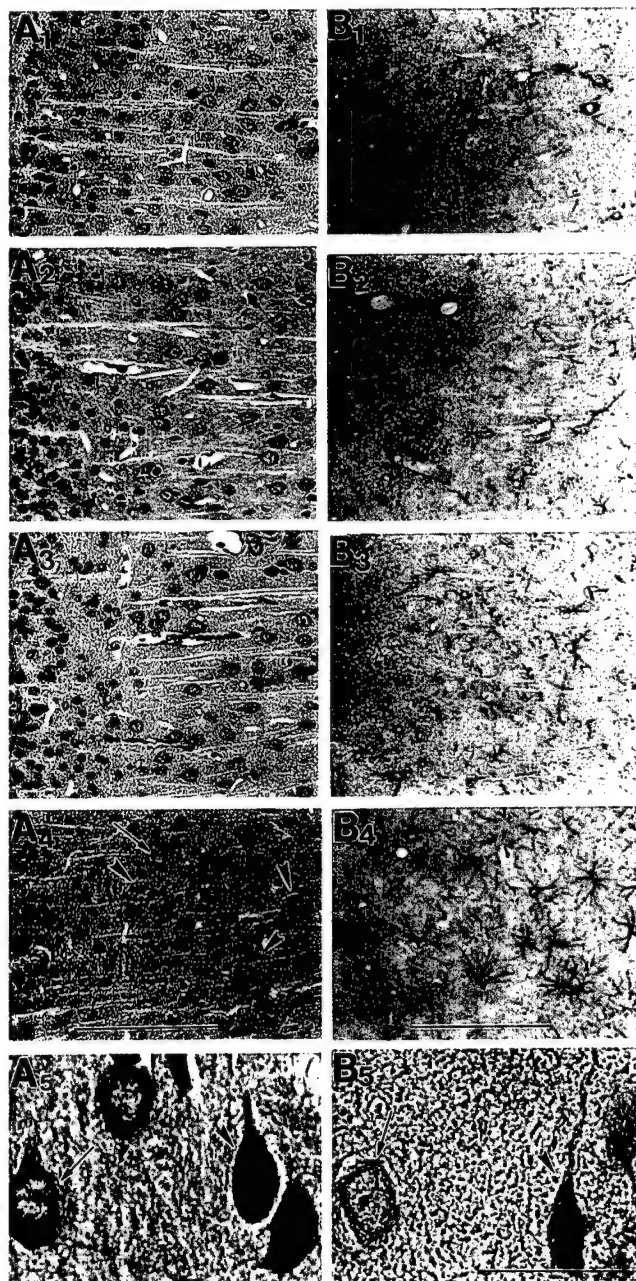


FIG. 8. Alterations in the cingulate cortex (layers II-V) following treatment with pyridostigmine bromide (PB), DEET, and permethrin, with and without stress. A1-A5, H&E staining; B1-B4, GFAP immunostaining. A1 and B1 are examples from a vehicle-treated control rat. A2 and B2 are examples from a rat subjected to stress alone. A3 and B3 are examples from a rat treated with chemicals alone. A4 and B4 are examples from a rat treated with both chemicals and stress showing neuronal cell death and an increased glial fibrillary acidic protein immunoreactivity. The arrowhead in A4 points to a dying neuron. A5 is a magnified view of a region from A4 showing both surviving (arrow on left side) and dying (arrowhead on right side) neurons. B5 illustrates a silver-stained section through the cingulate cortex from a rat treated with both

activity and morphology of brain capillary endothelial cells (Sarmiento *et al.*, 1990; Wisniewski *et al.*, 1997).

Previous studies have indicated that the BBB can be disrupted by stress (Friedman *et al.*, 1996) and environmental toxicants such as anti-cholinesterase compounds (Petralli *et al.*, 1991; Relyea & Mills, 2001). Damage to the BBB after stress is believed to be due to several factors. These include stimulation of central catecholaminergic neurons and release of noradrenaline (Bradbury, 1979; Rapoport *et al.*, 1980), an increased local activation and release of serotonin (Sharma *et al.*, 1991), changes in the circulating levels of corticosteroids (Barryd *et al.*, 1985), and an increased cerebral blood flow and energy metabolism (Bryan, 1990). Indeed, exposure to a significant acute or chronic stress causes neurological and psychological symptoms (Sunanda Rao & Raju, 1995; 2000; Esposito *et al.*, 2001). However, stress-induced BBB damage depends upon the type and the degree of stress and also the strain, species, and age of the animals (Fuchs & Flugge, 1998; Friedman *et al.*, 1996; Telang *et al.*, 1999). For example, certain types of immobilization stress can lead to significant BBB damage (Belova & Jonsson, 1982). A combination of swim stress and PB exposure can lead to an increased BBB permeability to PB and induce a significant decrease in AChE activity in FVB/N mice but not in Webster mice (Friedman *et al.*, 1996; Telang *et al.*, 1999). The contradictory results in the latter two experiments likely reflect differences in the vulnerability of the BBB between the two strains of mice, as there is some experimental evidence that FVB/N mouse strain have an unusually permeable BBB (Friedman *et al.*, 1996; Telang *et al.*, 1999).

In our study, a moderate amount of stress employed (i.e., restraint stress for 5 min/day for 28 days) did not induce noticeable BBB damage in rats subjected to stress alone. The only exception was in the thalamus, where a moderate HRP leakage (but not accompanied by a decrease in EBA-positive structures and neuronal cell death) was observed in the stress-alone group. Only the animals exposed to a combination of stress and chemicals exhibited a significant HRP leakage and rupture of the capillaries. A combined stress and chemicals exposure also induced a dramatic reduction

stress and chemicals. A dying pyramidal neuron in this sample (arrowhead on right) shows a dense staining in both soma and apical dendrite, in comparison to a lightly stained surviving neuron in the vicinity (arrow on left). Scale bar, A1-A4 and B1-B4 = 175 μ m; A5 and B5 = 20 μ m.

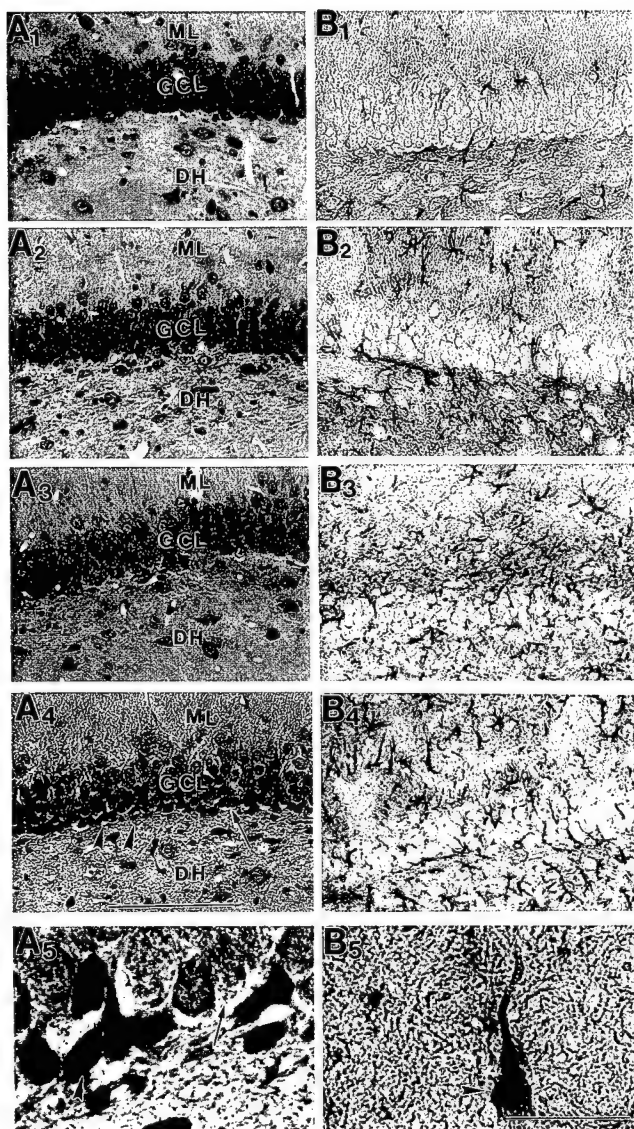


FIG. 9. Alterations in the dentate gyrus following treatment with pyridostigmine bromide (PB), DEET, and permethrin, with and without stress. A1–A5, H&E staining; B1–B4, GFAP immunostaining. A1 and B1 are examples from a vehicle-treated control rat. A2 and B2 are examples from a rat subjected to stress alone. A3 and B3 are examples from a rat treated with chemicals alone. A4 and B4 are examples from a rat treated with both chemicals and stress showing neuronal cell death and an increased glial fibrillary acidic protein immunoreactivity. Arrowheads in A4 point to dying neurons/cells in the dentate subgranular zone. A5 is a magnified view of a region from A4 showing both surviving neurons (with hematoxylin-stained large nucleus) and dying neurons (with condensed chromatin and eosin-stained soma and dendrites). B5 illustrates a silver-stained dying neuron within the dentate granule cell layer of a rat treated with both stress and chemicals. Scale bar, A1–A4 and B1–B4 = 175 μ m; A5 and B5 = 20 μ m.

in the expression of EBA. This is indicative of a marked alteration in the BBB, as a decrease in EBA staining reflects disruption of brain capillaries or degradation of the antigen (Sternberger & Sternberger, 1987; Mori *et al.*, 1992). However, the mechanisms of BBB damage following a combined exposure to stress and chemicals are not clear. It could be due to alterations in the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in the membrane protein of the cerebrovascular endothelial cells following exposure to a combination of stress and chemicals (Baker *et al.*, 1969; Corvette *et al.*, 1989). The latter alterations can cause vasodilatation and shrinkage of the endothelial cells, which in turn can lead to widening of the interendothelial tight junctions (Rapoport *et al.*, 1980). Further, calcium-mediated contraction of the endothelial cytoskeleton can open the interendothelial tight junctions (Rapoport, 2000), increase the intracellular calcium concentration, and affect the signal transduction pathways (Nagashima *et al.*, 1997). Additionally, permethrin-induced sustained opening of the sodium channels (Narahashi, 1985) may have also played a role in the BBB damage in this study. From the above, it appears that BBB disruption in many areas of the brain including the hippocampus following a combined exposure to stress and chemicals in this study reflect additive interaction of stress and chemicals. Coexposure to the stress and chemicals likely induces changes in the arrangement and topography of gap junction molecules and thereby allows opening of the junction and results in an increased BBB permeability. The biochemical events leading to the increased BBB permeability following exposure to stress and chemicals are unknown. However, it has been suggested that immediate early genes such as *c-fos* might play a role in stress-induced increases in BBB permeability (Melia *et al.*, 1994; Friedman *et al.*, 1996).

Regarding neuropathological changes in animals treated with stress and chemicals, the potential mechanisms might involve a synergistic interaction between stress and chemicals. This is because stress can cause BBB disruption due to the shrinkage of cerebral endothelial cells (Rapoport & Robinson, 1986) and induce the release of corticosteroids and amino acid receptor, which in regions rich in receptors for corticosteroids (such as the hippocampus and amygdala) can result in negative feedback regulation of the corticosteroids system (Magarinos & McEwen, 1995; Sapolsky, 1992; Brunson *et al.*, 2001) and reduced blood flow and oxygen (Magarinos & McEwen, 1995; Davids, 1981; Sapolsky, 1992). Chemicals, on the other hand, endanger excessive activity of ACh neurons once inside the brain (Friedman *et al.*, 1996; Grauer *et*

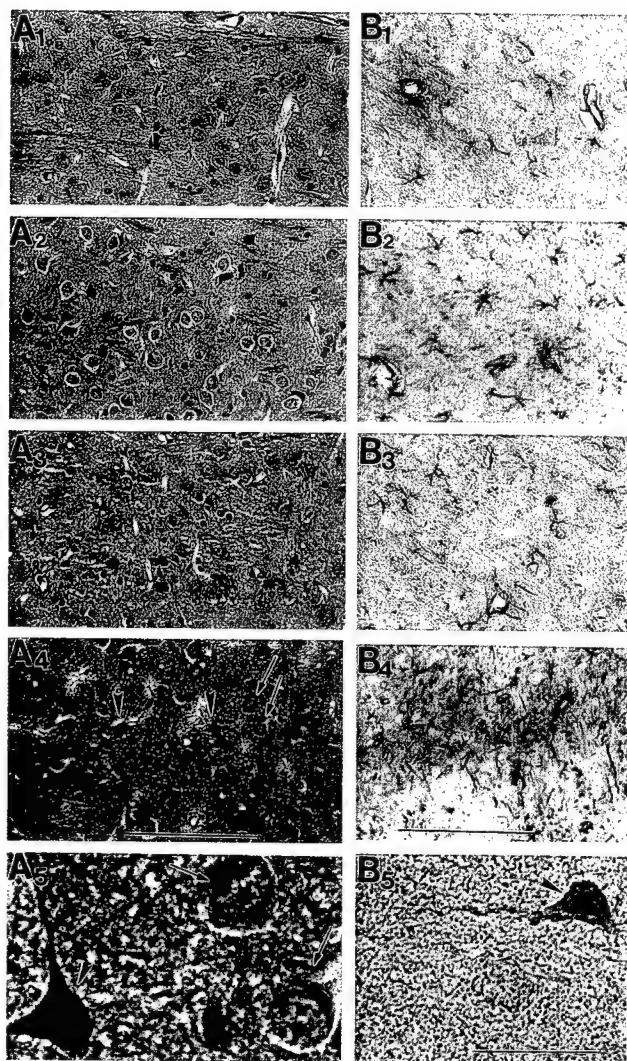


FIG. 10. Alterations in the lateral dorsal nucleus of the thalamus following treatment with pyridostigmine bromide (PB), DEET, and permethrin, with and without stress. A1–A5, H&E staining; B1–B4, GFAP immunostaining. A1 and B1 are examples from a vehicle-treated control rat. A2 and B2 are examples from a rat subjected to stress alone. A3 and B3 are examples from a rat treated with chemicals alone. A4 and B4 are examples from a rat treated with both chemicals and stress showing neuronal cell death (arrowhead in A4) and an increased glial fibrillary acidic protein immunoreactivity (B4). A5 is a magnified view of a region from A4 showing both surviving (arrow in upper right half) and dying (arrowhead on left side) neurons. B5 illustrates a silver-stained dying neuron within the lateral dorsal nucleus of the thalamus from a rat treated with both stress and chemicals; an arrowhead points to a surviving neuron close to the dying neuron. Scale bar, A1–A4 and B1–B4 = 175 μ m; A5 and B5 = 20 μ m.

al., 2001), which may result in an increased need for oxygen and blood flow. Thus, if the need for blood and oxygen exceeds the ability to supply it, death of neurons may ensue.

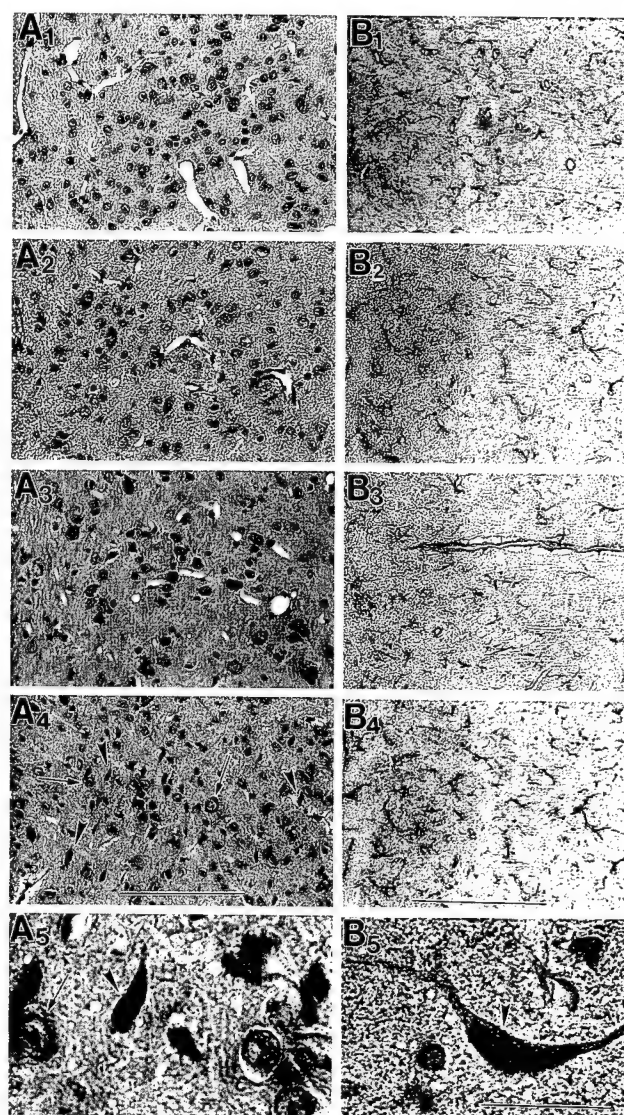


FIG. 11. Alterations in the dorsomedial nucleus of the hypothalamus after treatment with pyridostigmine bromide (PB), DEET, and permethrin, with and without stress. A1–A5, H&E staining; B1–B4, GFAP immunostaining. A1 and B1 are examples from a control rat. A2 and B2 are examples from a rat subjected to stress alone. A3 and B3 are examples from a rat treated with chemicals alone. A4 and B4 are examples from a rat treated with both chemicals and stress showing neuronal cell death (arrowhead in A4) and an increased glial fibrillary acidic protein immunoreactivity (B4). A5 is a magnified view of a region from A4 showing both surviving neurons with hematoxylin-stained large nucleus and a dying neuron with condensed chromatin and eosin-stained proximal dendrite (arrowhead). B5 illustrates silver-stained dying neurons within the dorsomedial nucleus of the hypothalamus of a rat treated with both stress and chemicals. Scale bar, A1–A4 and B1–B4 = 175 μ m; A5 and B5 = 20 μ m.

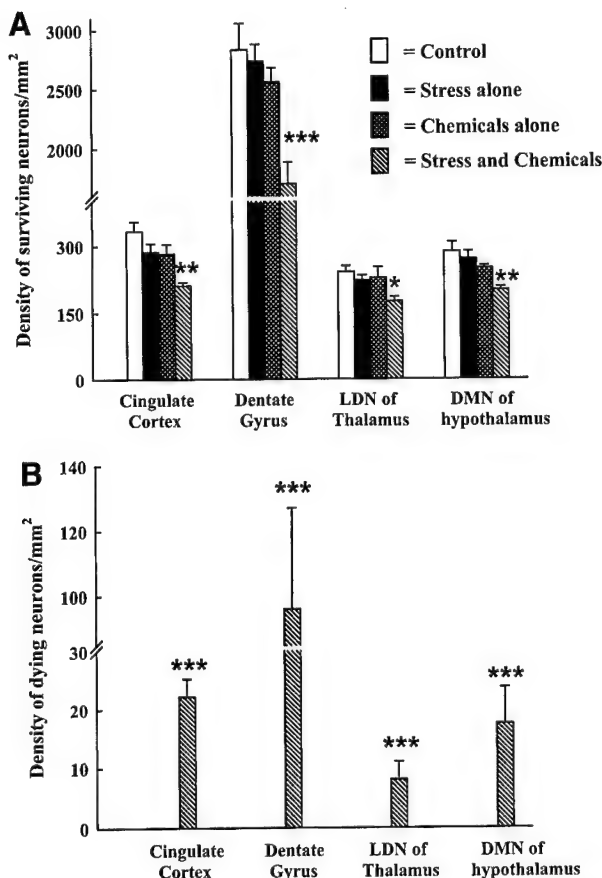


FIG. 12. Histograms show the density of surviving (A) and dying (B) neurons per square millimeter of the cingulate cortex, the dentate gyrus, the lateral dorsal nucleus (LDN) of the thalamus, and the dorsomedial nucleus (DMN) of the hypothalamus. Values represent means and standard errors ($n = 5/\text{group}$). Analyses with one-way ANOVA revealed significant differences between groups for both surviving neurons (cingulate cortex and DMN of the hypothalamus, $P < 0.01$; dentate gyrus, $P < 0.001$; LDN of the thalamus, $P < 0.05$) and dying neurons (all regions, $P < 0.001$). The post hoc analysis revealed that animals treated with stress and chemicals exhibited a significant decrease in the number of surviving neurons in comparison to vehicle-treated control animals and animals treated with either stress or chemicals alone. Further, animals treated with chemicals and stress exhibited a significantly increased number of dying neurons in all of the above regions, in comparison to both control animals and animals treated with either stress or chemicals alone. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Extent of Neuronal Cell Death after Exposure to Stress and Lower Doses of PB, DEET, and Permethrin

Previous studies have shown that exposure to higher doses of PB, DEET, and permethrin, alone or in combination, or subchronic exposure to lower doses of DEET and permethrin leads to significant brain dam-

age, characterized by neurological dysfunction, neuropathology, and behavioral abnormalities (Abou-Donia *et al.*, 1996, 2001; Abdel-Rahman *et al.*, 2001; Hoy *et al.*, 2000). In addition, several studies suggest that synergistic effects of low doses of PB, DEET, and permethrin lead to significantly decreased locomotor activity in both male and female rats (Abou-Donia *et al.*, 2001; Hoy *et al.*, 2000; Van Haaren *et al.*, 2001). The present results indicate that a combination of the stress and lower doses of the chemicals PB, DEET, and permethrin induces a significant neuronal cell death in all brain regions where BBB was also disrupted. In the cingulate cortex, the neuronal cell death was more pronounced in pyramidal neurons of layers III and V. In the dentate gyrus, neuronal cell death was conspicuous in the granule cells. In the thalamus and hypothalamus, neuronal cell death was consistently apparent in the lateral dorsal nucleus of the thalamus and the dorsomedial nucleus of the hypothalamus. The neuronal cell death in these regions was associated

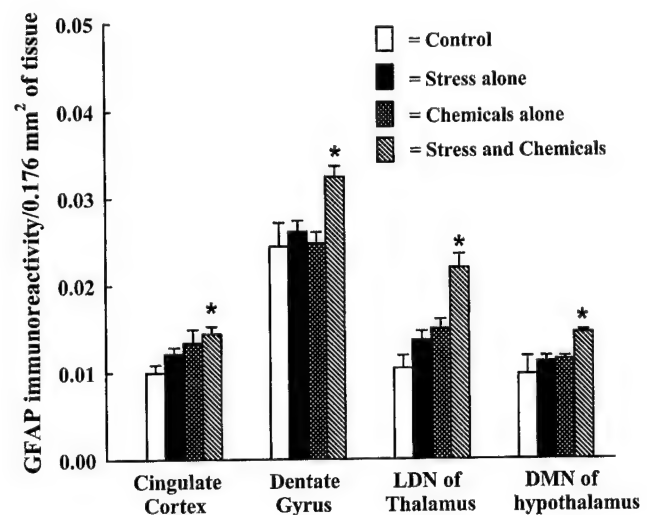


FIG. 13. Histograms show the area of GFAP-immunoreactive elements (in mm²) per unit area (0.176 mm²) of the cingulate cortex, the dentate gyrus, the lateral dorsal nucleus (LDN) of the thalamus, and the dorsomedial nucleus (DMN) of the hypothalamus. Values represent means and standard errors ($n = 5/\text{group}$). Analyses with one-way ANOVA revealed significant differences between groups (cingulate cortex, $P < 0.05$; dentate gyrus, $P < 0.01$; the LDN of the thalamus, $P < 0.001$; the DMN of the hypothalamus, $P < 0.05$). The post hoc analysis revealed that animals treated with stress and chemicals exhibited a significant increase (denoted by asterisks) in GFAP immunoreactivity, in comparison to vehicle-treated control animals. In comparison to animals treated with stress or chemicals alone, animals treated with both stress and chemicals revealed a significant increase in GFAP immunoreactivity within the dentate gyrus and the LDN of the thalamus. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

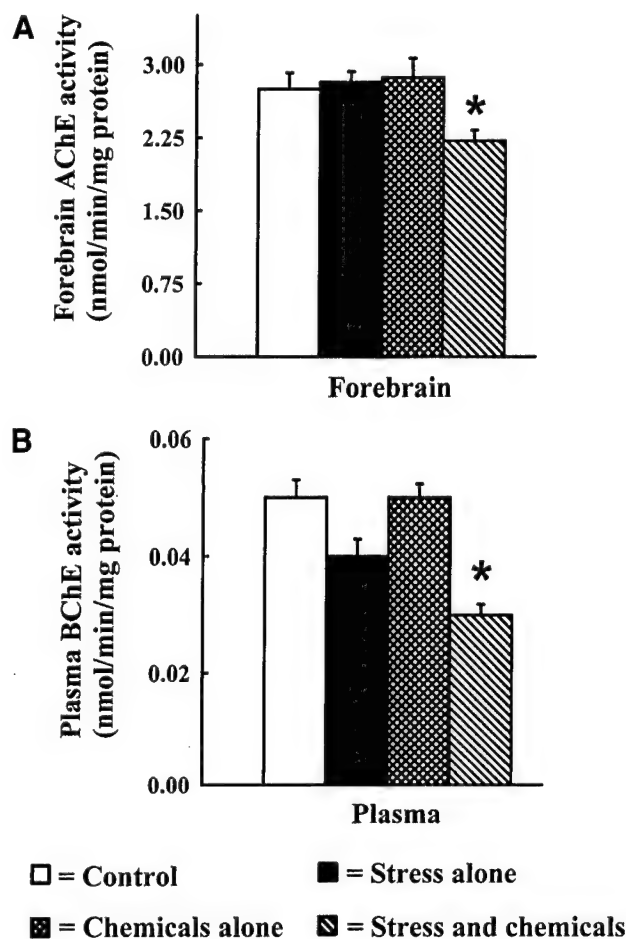


FIG. 14. Effect of administration of pyridostigmine bromide (PB), DEET, and permethrin with or without stress on forebrain AChE and (A) plasma BChE (B) activity. Values represent means and standard errors ($n = 5/\text{group}$). Analysis with one-way ANOVA revealed significant differences between groups in both the forebrain and the plasma. The post hoc analysis revealed that only animals treated with chemicals and stress exhibited a significant decrease (denoted by asterisks) compared to vehicle-treated controls in both the forebrain and the plasma. Further, compared to animals treated with stress alone, BChE activity in plasma showed a significant decrease in animals treated with both chemicals and stress. $*P < 0.05$.

with an increased GFAP immunoreactivity, which is also suggestive of the presence of brain injury (Dickson *et al.*, 1993; Norenberg, 1994).

Thus, a pronounced neural injury occurs following exposure to both stress and chemicals compared to either stress or chemicals alone. The differential neural damage between these groups likely reflects differences in the permeability of BBB following various treatments. As exposure to both chemicals and stress

significantly increases BBB permeability and liver damage, the ability of these rats for the detoxification of chemicals is compromised significantly, resulting in a much greater concentration of chemicals crossing the BBB and reaching different target sites in the brain (Abou-Donia *et al.*, 1996). A significant inhibition of brain AChE in the forebrain and liver injury observed in this study supports the above contention.

Potential Reasons for Changes in AChE Activity after Exposure to Both Stress and Chemicals

Among different chemicals used in this study, PB is known to inhibit the peripheral cholinesterase but not brain AChE, as it does not normally cross the BBB. In this study, even when treated with other chemicals, PB did not decrease brain AChE, suggesting a lack of PB penetration across the BBB under these conditions. However, when combined with stress and other chemicals, there was a significant decrease in brain AChE and m2-muscarinic Ach receptors, suggesting that PB is capable of entering the brain when combined with stress and other chemicals. It is possible that PB enters the CNS parenchyma because of the

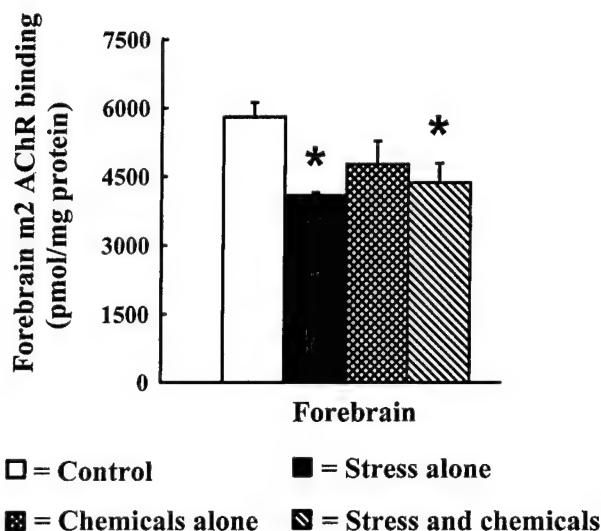


FIG. 15. Effect of the administration of pyridostigmine bromide (PB), DEET, and permethrin with or without stress on brain m2-muscarinic receptor ligand binding in the forebrain. Analysis with one-way ANOVA revealed significant differences between groups ($P < 0.05$). The post hoc analysis revealed that animals treated with chemicals and stress exhibited a significant decrease (denoted by asterisks) compared to vehicle-treated controls. Further, in animals treated with stress alone, BChE activity in plasma showed a significant decrease (denoted by asterisks), in comparison to vehicle-treated control animals. $*P < 0.05$.

combined effect of stress and the chemicals PB, DEET, and permethrin on BBB permeability. Indeed, several studies demonstrate that a significant inhibition of cholinesterase in the brain capillary walls by cholinesterase inhibitors can significantly alter the BBB permeability (Grauer *et al.*, 2001; Skultetyova *et al.*, 1998). Thus, in this study, it appears that disruption of the BBB (induced by a combined effect of stress and chemicals) caused the entry of PB into the brain, and the consequent inhibition of brain AChE activity and regulation of its receptors by PB enhanced the neurotoxic effects of the chemicals. Thus, exposure to PB in the presence of other neurotoxic agents and stress can lead to significant damage to the brain.

Concluding Remarks

There have been two different hypotheses for the basis of the Gulf-War syndrome, one of which is focused predominantly on "stress" as the cause, whereas the other theory is that Gulf-War syndrome is a result of exposure to a wide variety of chemicals including pesticides, the prophylactic drug PB, other experimental vaccines, depleted uranium, and the nerve gas sarin (IOM, 1997; Shays, 1997; Metcalf, 1997). In this study, using adult rats, we rigorously investigated the neuropathological effects of a combined exposure to a moderate level of stress and low doses of selected chemicals (PB, DEET, and permethrin) to which PGW veterans were believed to be exposed during the PGW. In addition, the route of exposure and dose levels of test compounds in this study were approximately equivalent to the exposure that may have occurred to army personnel during the Gulf War (Institute of Medicine, 1995; W. McCain Department of Defense, personal communication). Our results reveal that a combined exposure to stress and chemicals causes both disruptions of the BBB and neuronal cell death in many regions of the brain. This is in great contrast to animals exposed to either stress or chemicals alone, which remain mostly comparable to vehicle-treated control animals. These results underscore that combined exposure to moderate stress and low doses of the chemicals PB, DEET, and permethrin leads to significant brain injury. In this context, it is likely that the various neurological symptoms (such as memory loss, dizziness, impaired sense of direction, depression, and amyotrophic lateral sclerosis) reported by PGW veterans is, at least, partially linked to this kind of brain injury incurred during the war.

ACKNOWLEDGMENTS

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TESTICULAR GERM-CELL APOPTOSIS IN STRESSED RATS FOLLOWING COMBINED EXPOSURE TO PYRIDOSTIGMINE BROMIDE, *N,N*-DIETHYL *m*-TOLUAMIDE (DEET), AND PERMETHRIN

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*This study reports and characterizes the testicular apoptosis following daily exposure of male Sprague-Dawley rats to subchronic combined doses of pyridostigmine bromide (PB, 1.3 mg/kg/d in water, oral), a drug used for treatment of myasthenia gravis and prophylactic treatment against nerve agents during the Persian Gulf War; the insect repellent *N,N*-diethyl *m*-toluamide (DEET, 40 mg/kg/d in ethanol, dermal); and the insecticide permethrin (0.13 mg/kg in ethanol, dermal), with and without stress for 28 d. Combined exposure to these chemicals was implicated in the development of illnesses including genitourinary disorders among many veterans of the Persian Gulf War. Previous studies from this laboratory have shown that exposure to combination of these chemicals produced greater toxicity compared to single components. Exposure to stress alone did not cause any significant histopathological alterations in the testes. Administration of combination of these chemicals induced apoptosis in rat testicular germ cells, Sertoli cells, and Leydig cells, as well as in the endothelial lining of the blood vessels. Testicular damage was significantly augmented when the animals were further exposed to a combination of chemicals and stress. Histopathological examination of testicular tissue sections showed that apoptosis was confined to the basal germ cells and spermatocytes, indicating suppression of spermatogenesis. Increased apoptosis of testicular cells coincided, in timing and localization, with increased expression of the apoptosis-promoting proteins Bax and p53. Furthermore, significant increase of 3-nitrotyrosine immunostaining in the testis revealed oxidative and/or nitrosation induction of cell death. In conclusion, combined exposure to real-life doses of test compounds caused germ-cell apoptosis that was significantly enhanced by stress.*

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Concurrent exposure to pyridostigmine bromide (PB), the insect repellent *N,N*-diethyl *m*-toluamide (DEET), and the pyrethroid insecticide permethrin has been suggested as a possible cause of Persian Gulf War Illnesses in service personnel (Abou-Donia et al., 1996a, 1996b; McCain et al., 1997). In addition to causing neurological disorders (Abdel Rahman et al., 2001, 2002), combined exposure to these chemicals increased urinary excretion of 8-hydroxy-2'-deoxyguanosine and 6- β -hydroxycortisol (Abu-Qare & Abou-Donia, 2000, 2001a) and decreased permeability of the blood-testes barrier (BTB) (Abou-Donia et al., 2001). Some veterans also suffered from genitourinary disorders and increased occurrence of neoplasms, among which was testicular cancer (Knocke et al., 1998; Gray et al., 1996). Also, stress augmented the action of the three test compounds, in the same animals, in increasing the permeability of the blood barrier and causing neuronal cell death in the cingulate cortex, dentate gyrus, thalamus, and hypothalamus in rat brain (Abdel-Rahman et al., 2002). Recently, pyrethroid insecticides have been shown to inhibit the activity of respiratory-chain enzymes of mammalian mitochondria (Gassner et al., 1997) and increased release of brain mitochondrial cytochrome c (Abu-Qare & Abou-Donia, 2001b). This disturbance of the mitochondrial respiratory chain could provide an explanation for the pathological effects associated with pyrethroid intoxication.

Apoptosis, or programmed cell death (PCD), is defined by characteristic morphological and molecular events that result in the efficient elimination of cells from a tissue without eliciting an inflammatory response (Kerr et al., 1972; Wyllie et al., 1980). The process of apoptosis is involved in development of homeostatic regulation of cell populations and in the pathogenesis of certain diseases (Thompson, 1995; Abu-Qare & Abou-Donia, 2001c). Elimination of germ cells via apoptosis occurs spontaneously under normal physiologic conditions and is often amplified by testicular injury. Studies by Flemming (1887) are widely cited as being the first to describe the occurrence of spontaneous germ-cell degeneration in the testes under normal physiologic conditions. Studies in the late 1980s, and early 1990s, however, recognized this physiologic condition of germ-cell degeneration as apoptosis (Allan et al., 1992; Tapanainen et al., 1993; Billig et al., 1995; Brinkworth et al., 1995). Although the physiological occurrence of apoptosis in the testes has been described in detail, the molecular and cellular mechanisms regulating this process are not well understood.

Germ-cell apoptosis has been demonstrated in response to androgen withdrawal (Tapanainen et al., 1993) and heat stress (Orr & Mann, 1992; Sapolsky, 1985). Reproductive function in male mammals is suppressed by psychogenetic or somatic stress (Sapolsky, 1985; Charpenet et al., 1982). In rats, restraint-induced stress usually lowers plasma testosterone concentrations (Charpenet et al., 1982). Although studies are available on the effect of toxicants on testicular toxicity (Somkoti et al., 1987), there is no literature dealing with the relationship of stress combined with environmental chemicals to testicular germ-cell apoptosis.

In the present study, an immunohistochemical method based on the detection of apoptotic cells with a monoclonal antibody to single-stranded (ss) DNA was applied for the analysis of apoptosis in rat testicular tissue. The aim was to determine the possible association of the apoptosis in these injured testes with the histopathological appearance and Bax and p53 expression following coexposure to real-life doses of DEET, permethrin, and PB, with and without stress.

MATERIALS AND METHODS

Chemicals

Technical-grade (93.6%) permethrin [(±)-*cis/trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid (3-phenoxyphenyl)methyl ester] was obtained from Roussel Uclaf Corp., Pasadena, TX. DEET (≥97% *N,N*-diethyl *m*-toluamide), pyridostigmine bromide (99% 3-dimethylamino carbonyloxy-*N*-methylpyridinium bromide, PB), and diaminobenzidine tetrahydrochloride were purchased from Sigma Chemical Co., St. Louis, MO. Monoclonal antibodies to ssDNA were obtained from Chemicon International, Temecula, CA. Polyclonal antibodies to Bax were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Mouse monoclonal antibodies to p53 were obtained from Oncogene, Cambridge, MA. Antibodies against 3-nitrotyrosine were obtained from Zymed Laboratories, San Francisco, CA. Polyclonal antibodies to glial fibrillary acidic protein (GFAP) were purchased from Dako Corporation, Carpinteria, CA. Avidin-biotin-peroxidase detection reagent was obtained from Vector Laboratories, Burlington, CA. All other chemicals and reagents were of highest purity available from commercial sources.

Animals

Male Sprague-Dawley rats (225–250 g) were obtained from Zivic-Miller Laboratories, Allison Park, PA. The animals were randomly assigned to control and treatment groups and housed at 21–23 °C with a 12-h light/dark cycle. They were supplied with Purina certified rodent chow (Ralston Purina Co., St. Louis, MO) and tap water ad libitum. The rats were allowed to adjust to their environment for a week before treatment. Animal care was in accordance with the U.S. Army institutional guidelines and was approved by the Duke University Animal Care and Use committee.

Treatment

Four groups of rats (10 animals per group) were treated daily with the 3 test compounds, alone or with stress or vehicle, for 28 d. A daily dose of 1.3 mg/kg pyridostigmine bromide (PB) was dissolved in water (1 ml/kg) and administered orally using a round-head, 3-cm needle syringe (Popper & Son, Inc., New Hyde Park, NY). Daily dermal application of 40 mg/kg DEET and 0.13 mg/kg permethrin, each dissolved in 70% ethanol (1 ml/kg), was administered

with a micropipette to a separate unprotected 1-cm² areas of preclipped skin on the back of each animal's neck. The dosages chosen were representative of human exposure levels (Abou-Donia et al., 2001). Stress was carried out by placing each rat in a Plexiglas restrainer for 5 min daily. Animals were treated according to the following schedule:

- Group A. Control: water (1 ml/kg) orally and 70% ethanol (1 ml/kg) dermally,
- Group B. Stress alone,
- Group C. Chemical combination: PB, DEET, and permethrin,
- Group D. Chemicals+stress: PB, DEET, permethrin, and stress.

Histopathological Assessment

Five animals from each control and treatment groups were anesthetized with 50 mg/kg sodium pentobarbital. Each animal was preserved by transcardiac perfusion with saline, followed by 4% paraformaldehyde and 0.1% glutaraldehyde in Tris-buffered saline (TBS, pH 7.4). Testes were removed, postfixed, dehydrated in graded ethanol, and embedded in paraffin. Sections (3–5 μ m) were stained with hematoxylin and eosin (H&E) for microscopic examination, as well as with specific cellular markers described later.

Single-Stranded DNA Detection

This method is a specific and sensitive cellular marker for detection of apoptosis independent of internucleosomal DNA fragmentation and useful for the detection of different stages of apoptosis (Frankfurt et al., 1996). More importantly, in contrast with the TUNEL method, staining with ssDNA monoclonal antibody (mAb) clearly differentiates between the apoptotic and necrotic mechanisms of cell death. Briefly, paraffin-embedded sections were incubated at 60 °C in formamide (50%) for 30 min, followed by washing in ice-cold phosphate-buffered saline (PBS) as described by Frankfurt et al. (1996). The procedure was then carried out as standard immunohistochemical staining using mAb against ssDNA as 1:100 dilution (Chemicon International, Temecula, CA). The apoptotic indices were determined by microscopic examination of a total of 10 randomly selected seminiferous tubules at high-power fields ($\times 400$). In each specimen, at least 2000 cells were evaluated for the number of nuclei exhibiting apoptotic staining.

Immunohistochemistry

Three- to 4- μ m-thick tissue sections were dewaxed in xylene and rehydrated through graded alcohols. Slides were heated in 10 mmol/L sodium citrate buffer (pH 6) in a microwave oven (750 W) 3 times for 5 min. Primary antibodies against 3-nitrotyrosine (mouse monoclonal, Zymed Laboratories, San Francisco, CA, diluted 1:100 in Tris-buffered saline), Bax (rabbit polyclonal anti-Bax, N-20, Santa Cruz Biotechnology, Santa Cruz, CA, 1:50), and p53 (mouse monoclonal anti-p53, Oncogene, Cambridge MA, 1:20) were applied for 2 h at room

temperature, respectively. After washing in Tris-buffered saline, sections were incubated for 45 min with appropriate biotinylated secondary antibodies (Zymed Laboratories, San Francisco, CA) and then with streptavidin-horseradish peroxidase conjugate (Zymed) for 15 min. Antigenic sites were revealed by incubating sections in 0.05% 3,3'-diaminobenzidine in Tris saline with hydrogen peroxide as a substrate. After washing in tap water, the sections were counterstained with nuclear fast red, dehydrated through graded alcohols, cleared in xylene, and mounted with DePeX.

Statistical Analysis

Statistical differences among groups were tested by analysis of variance (ANOVA) with Fisher post hoc analysis using commercially available software (Stat view 4.01). A *p* value of .05 was considered significant.

RESULTS

All treated animals appeared healthy during the 28-d experiment. At the end of the experiment, only rats in group D (Chemicals+stress) gained significantly less weight (~14% less) than the control group, whose percentage of initial body weight (mean \pm SEM) was 174 ± 1.22 , compared to 172 ± 1.50 for stressed animals, and 173 ± 1.71 for the chemicals-treated group.

Histological Characteristics

Histopathological assessments were performed on testicular sections from control and treated rats. The hematoxylin and eosin (H&E)-stained testes sections from control rats revealed the presence of normal seminiferous tubules undergoing spermatogenic cycle composed of spermatogonia, spermatocytes, round spermatids, and elongated spermatids consistent with intact spermatogenesis (Figure 1A). In sections from stress-treated animals, the germ cells and Sertoli cells within the seminiferous tubules were normal, as were the interstitial (Leydig) cells (Figure 1B). The major histologic abnormalities were observed in the chemically treated rat testes with or without stress. The most prominent pathological features observed in the chemically intoxicated rats were arrested spermatogenesis and occasional seminiferous tubular degeneration, edema of the interstitial surroundings, and thickening of the interstitium (Figure 1C). In rats treated with combination of the three chemicals plus stress (Figure 1D), the most prominent pathological feature observed was arrested spermatogenesis and frequent seminiferous tubular degeneration. Further, 27.4% of the tubules showed marked loss or complete loss of the spermatocytes and spermatids. Additionally, stress exacerbated the effects of chemicals resulting in varying degrees of germ cell degenerative changes, ranging from loss of some of the elongated spermatids, disorganization of germ cell layers, detachment, and sloughing to vacuolization of the seminiferous tubules, contributing to eventual atrophy. Furthermore, in the testes from rats treated with chemicals and stress (group D), most of the developing stages of the sperm

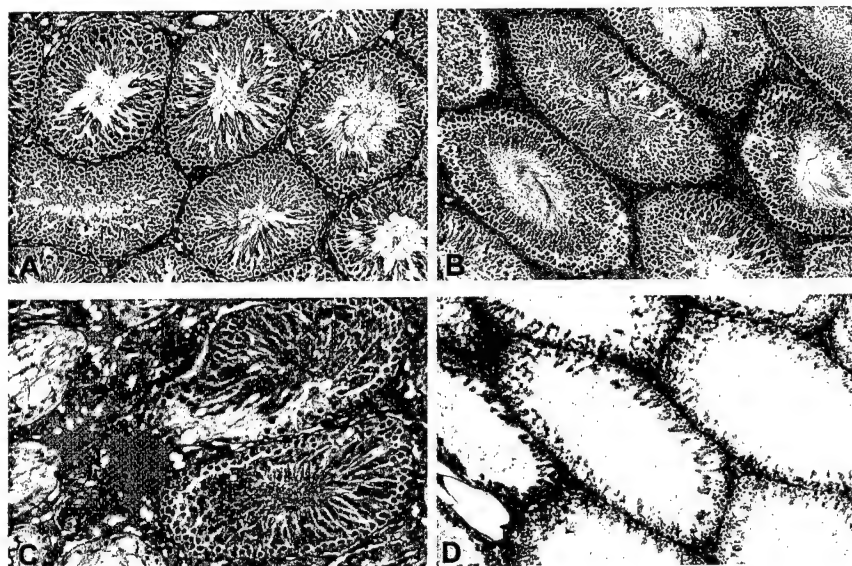


FIGURE 1. Photomicrographs of rat testes cross sections. (A) Section from the control rats testes show normal testicular tissue structure (hematoxylin and eosin, H&E). (B) Testes from stress-treated groups shows normal testes tubular structure with mild thickening of the interstitium (*). (C) Section from combined chemicals treated rats shows arrested spermatogenesis, thickened interstitium, vacuolization, and edema (*). (D) The micrograph is representative of rat testes exposed to three chemicals plus stress showing disruption of spermatogenesis and depleted cellularity of the atrophic seminiferous tubules. Spermatozoa are not present in the luminal aspect of these tubules. In (C) and (D), some tubules, dyscohesive basal germ cells, and desquamated cells were shed into the lumen of seminiferous tubules. Basal germ cells and primary and secondary spermatocytes show typical features of apoptosis as cell nuclei have dark, condensed, and fragmented chromatin matter and the cells appear smaller in size. In (D), note the lack of inflammatory cells (H&E, 240 \times).

were interrupted and some of the stages were absent. In this group, the abnormality of seminiferous tubules was also variably expressed; similar findings were noted in animals in group C, that were chemically treated. The percentage of atrophic tubules was (mean \pm SD) $0.88\pm0.07\%$, 9.8 ± 3.6 , and $27.4\pm8.3\%$ in stress, chemicals, and combined stress and chemicals, respectively. No inflammatory reaction was present. Despite the severe effect noticed in the seminiferous tubules, no inflammatory cells were observed in interstitial tissues or in the seminiferous tubules. Table 1 summarizes the results obtained from the histopathological evaluations following different exposures. The histological assessments indicated significant damage induced by exposure to combination of chemicals with or without stress. The percentages of normal tubules were markedly decreased after exposure to three chemicals alone. Greater incidence of detachment and sloughing was also evident. The highest incidence of

TABLE 1. Histological Assessment of Testicular Germ Cell, 28 d Following Exposure to Repetitive Administration of DEET, Permethrin, and PB With or Without Physical Stress

Treatment	Percentage of tubules			
	Normal	Detached	Sloughed	Vacuolized
Control	98.3 ± 1.2 ^a	0.9 ± 0.1	0.0	0.0
Stress	96.8 ± 1.6 ^a	1.6 ± 0.5	0.0	0.0
Chemicals	78.5 ± 3.8	10.2 ± 3.2 ^a	9.8 ± 2.1 ^a	2.5 ± 0.4
Chemicals + stress	71.8 ± 5.6	14.6 ± 3.8 ^a	12.4 ± 2.6 ^a	1.6 ± 3.2 ^a

Note. Means ± SE from five testes per group. Each histopathological endpoint was scored by evaluating 50–100 seminiferous tubules in the sections of each testis.

^a Significantly different from control and other groups ($p < .05$).

vacuolization was evident in testes from rats treated with chemicals only; 2.5% of the tubules showed vacuolization with marked loss or almost complete loss of spermatocytes and spermatids.

In Situ Assessment of Apoptosis

To determine whether the chemically induced germ-cell degeneration occurs via apoptosis, the extent of cell death by apoptosis was assessed in situ using antibody against single-stranded DNA. In control rats the testes showed a normal architecture of the seminiferous epithelium with only slight signs of apoptosis. Labeling was limited to a small number of sperm in the lumen of the seminiferous tubules (Figure 2A). In quantitative analysis of degenerating germ cells and spermatocytes with or without ssDNA labeling, the highest labeling signals were observed in animals treated with chemicals only (group C) and chemicals and stress-treated animals (group D) (Figure 2, C and D). In the testes from rats in group D, however, the number of apoptotic germ cells was underrepresented because of detection of only residual germ cells or fragmented DNA lining the tubules (Figure 2D). Nuclear staining for ssDNA was seen in some of the interstitial and somatic cells. There was no staining of germ cells when ssDNA antibody step was omitted from the sections. Based on cellular location within the seminiferous tubule and nuclear morphology, the majority of apoptotic cells was identified as primary spermatocytes (Figure 2, C and D). The number of immunolabeled cells in the seminiferous tubules was compared between groups (Table 2). The number of immunopositive cells was significantly higher in the groups treated with chemicals (C and D) compared with the control and stressed rats. The percentages of apoptotic cells in the rats from groups C and D were about 7-fold and 10-fold higher, respectively, than in the control. The preferential loss of mature germ cells within the lumen of testes sections obtained from animals in groups C and D revealed a selective susceptibility of these cells to combination of PB, DEET and permethrin-mediated cytotoxicity that was exacerbated by stress.

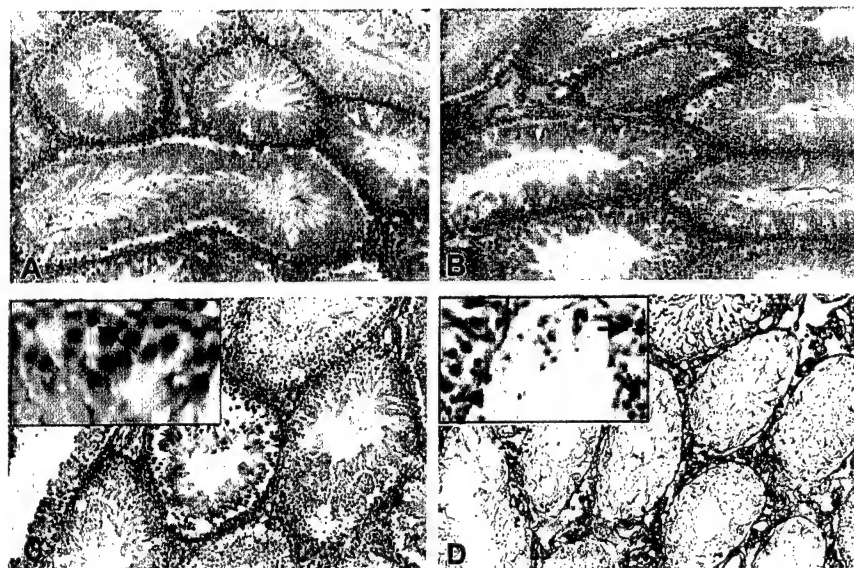


FIGURE 2. Micrographs of ssDNA-stained seminiferous tubules. (A) Section from control rat testes stained with mAb against ssDNA shows few sperms in the lumen of the seminiferous tubules are apoptotic. (B) Sections from rat testes treated with stress only shows apoptosis in small number of germ cells, spermatocytes, and few spermatozoan cells. (C) Sections from rat testes treated with combination of three chemicals, showing increased number of apoptotic cells, including germ cells, spermatocytes, and round spermatids. Fewer number of elongated spermatids showed mild immunoreactivity. The inset is a higher magnification ($\times 500$) showing positive nuclear staining cells (arrow). (D) Section from rat testes treated with combinations of chemicals plus stress. Note the empty seminiferous tubules and increased number of apoptotic nuclei in interstitial cells and blood vessel lining endothelial cells. Most of the seminiferous tubules were degenerated with complete loss of cellularity. The inset is a higher magnification ($\times 400$) showing positively stained apoptotic cells (arrow) and positively stained apoptotic germ cells in empty tubule (arrowhead). All other magnifications are $\times 240$.

TABLE 2. Percentage of Testicular Germ Cell Apoptosis 28 d Following Exposure to Combined Repetitive Administration of DEET, Permethrin, and PB With or Without Stress

Treatment	Mean percentage of tubules with apoptotic nuclei	Mean percentage of apoptotic nuclei
Control	3.5 ± 1.3	1.8 ± 0.2
Stress	8.3 ± 1.8	3.5 ± 0.4
Chemicals	22.8 ± 3.2^a	66.4 ± 4.8^a
Chemicals + stress	34.9 ± 5.6^b	79.6 ± 6.2^b

Note. Values represent the means \pm SE from five testes per group. Apoptotic cells were scored by evaluating 50–100 seminiferous tubules in sections of each testis.

^aSignificantly different from controls, $p < .05$.

^bSignificantly different from each other, $p < .05$.

3-Nitrotyrosine Staining

There was no positive 3-nitrotyrosine staining in rats from control group (Figure 3A). Fewer cells exhibited 3-nitrotyrosine staining in testes from rat exposed to stress only (Figure 3B), while increased immunoreactivity was detected in the animals treated with the three chemicals (Figure 3, B and C). This staining was considered to be specific because it was abolished by omitting the antibody from the staining procedure (data not shown). In contrast, immunohistochemical analysis of sections obtained from rats treated with combined chemicals plus stress revealed positive staining for 3-nitrotyrosine (Figure 3D), which was primarily cytoplasmic with some nuclear staining. Staining was localized in the spermatogonia, primary spermatocytes, and secondary spermatocytes and spermatids. Cytoplasmic and nuclear staining of the parenchymal and vessel cells interstitium was noted.

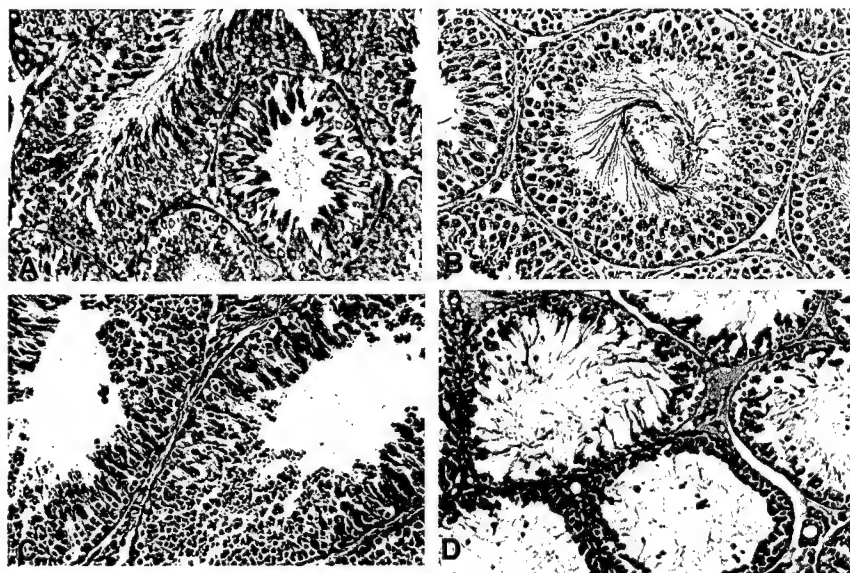


FIGURE 3. Immunoreactivity of 3-nitrotyrosine (3-NT) in rat testes. (A) Lack of immunoreactivity of 3-NT in control rat testes. (B) Fewer spermatozoan cells are staining for 3-NT in testes from rats exposed to stress only. (C) Testes from rats intoxicated with combination of three chemicals showing multi cell types stained positively for 3-NT. Note increased cytoplasmic staining for 3-NT in the germinal cells. (D) Immunoreactivity of 3-NT in testicular tissue from rats intoxicated with combination of three chemicals plus stress. The germ cells, primary spermatocytes, secondary spermatocytes, and spermatids are showing cytoplasmic and some nuclear staining. Limited staining of the interstitium including the blood vessels is evident in the area (arrow) ($\times 240$).

Expression of Apoptosis-Related Gene Products

The pro-apoptotic gene products examined in this study were Bax and p53. Using immunohistochemical analyses, Bax (Bcl family member) and p53 showed varying extents of immunostaining within the seminiferous tubules and the interstitium. While Bax protein expression was absent in control rat testes (Figure 4A), fewer cells immunostained for Bax protein were seen in the stressed rat testes (Figure 4B). In contrast, strong immunostaining was evident within the seminiferous epithelium and the interstitium from animals exposed to combined chemicals with or without stress (Figure 4, C and D). The p53 protein expression was barely detected in the seminiferous tubules from stressed rats (Figure 5B). In testes from combined chemicals-treated rats with or without stress, p53 expression was found primarily in the nuclei of spermatogonia, spermatocytes, and Sertoli cells (Figure 5, C and D). Occasionally, some interstitial cells were also positively immunostained for Bax and p53. In the control experiments, the immunoreactivities of these proteins (Bax and p53) were totally abolished when the primary antibodies were preabsorbed by their corresponding peptides (100-fold excess amount of the antibodies was

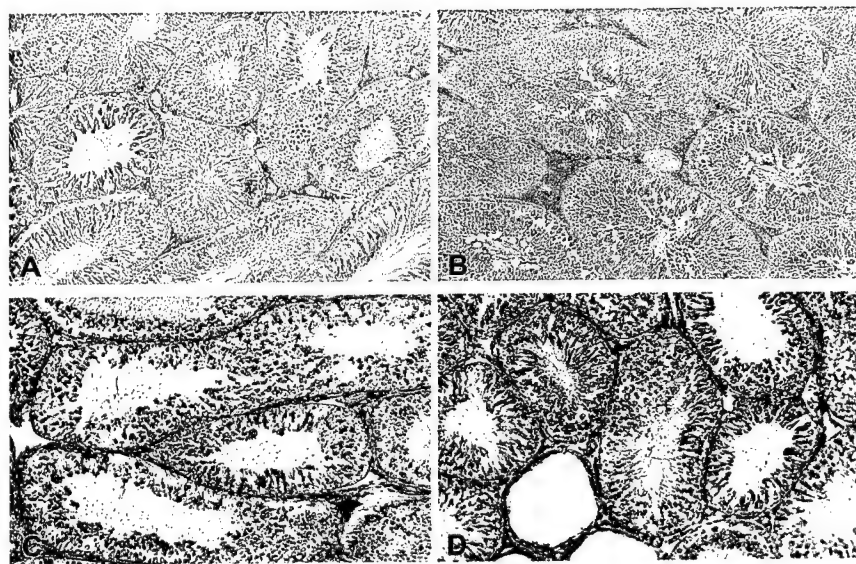


FIGURE 4. Expression of Bax in rat testes. (A) Lack of Bax expression in normal control rat testes. (B) Fewer spermatozoan cells are staining for Bax in testes from rats exposed to stress only. (C) Testes from rats intoxicated with combination of three chemicals. Multi cell types were showing increased staining with Bax antibody, including germinal and interstitial cells. (D) Increased cytoplasmic staining of Bax in the germinal cells of testes from rat intoxicated with combination of three chemicals plus stress. High intensity of staining for Bax is evident in both the germinal cells and interstitial cells ($\times 240$).

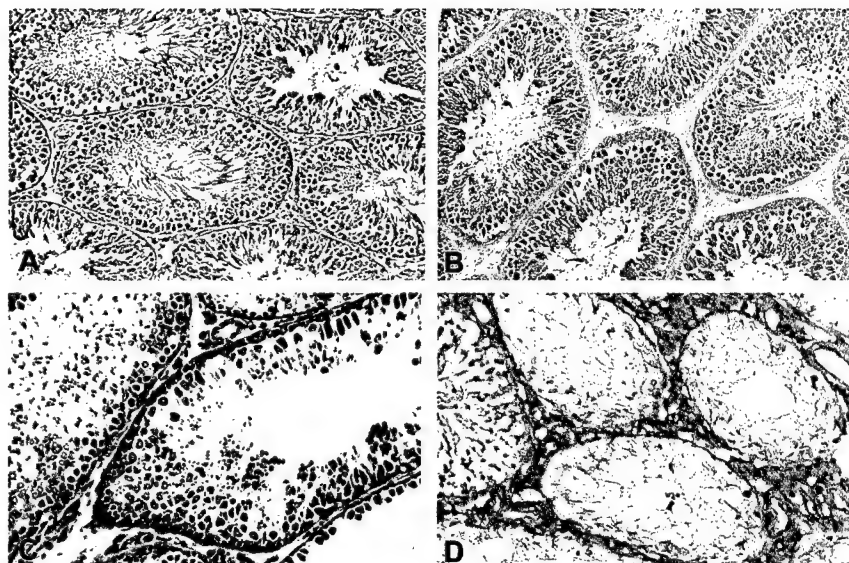


FIGURE 5. Expression of P53 in rat testes. (A) Lack of p53 expression in normal control rat testes. (B) Very mild immunoreactivity was seen in some spermatozoan of seminiferous tubules from stressed rat testes. (C) Testes from rats intoxicated with combination of three chemicals. Sertoli cells and interstitial cells are also showing both cytoplasmic and nuclear immunoreactivity. (D) In testes from rats intoxicated with combination of the three chemicals plus stress, note decreased cellularity. The remaining number of germinal cells and interstitial cells showed increased cytoplasmic and nuclear staining for p53 (260x).

used), while the immunostaining patterns did not change when the primary antibodies were treated with an unrelated peptides (data not shown).

DISCUSSION

This study demonstrates that a combination of PB, DEET, and permethrin is a potential testicular toxicant producing germ-cell apoptosis that was significantly augmented by stress. DEET tested alone did not alter sperm count, sperm morphology, or sperm viability in rats (Lebowitz et al., 1983). The combination of the three chemicals, however, may have synergistic and/or additive effects resulting in severe testicular damage. The data presented in the present study have identified apoptosis as the major cause of cell death in testicular tissues of chemical-treated rats, and this damage was enhanced by exposing animals to stress. Similarly, Abu-Qare and Abou-Donia (2001b) showed that DEET and permethrin, alone or in combination, induced apoptosis in rat brain. Male rats treated with testosterone inhibitors showed increased degeneration of Leydig cells and germ cells by apoptosis (Troiano et al., 1994). A transient increase in serum corticosterone and a transient decrease in serum testosterone were

observed during immobilization stress, which was the cause of enhanced apoptosis (Yazawa et al., 1999). These findings suggest that stress can augment testicular germ-cell apoptosis in rats exposed to combined chemicals.

In the present study, histological examination of testicular tissue sections from rats treated with chemical mixture plus stress showed that apoptosis was confined to the basal germ cells and primary and secondary spermatocytes. These changes, in addition to the appearance of Sertoli-cell vacuoles in the chemically treated animals, point to suppression of spermatogenesis. Such features are likely the result of the failure of Sertoli cells to support older germ cells, or the death of the more mature cells that are subsequently phagocytosed. Additionally, some of these chemicals might also affect Sertoli cell functions. Thus, the increase in apoptosis in germ cells, particularly with stress plus chemical exposure, might result not only from a direct effect of germ cells proliferation but also from alterations of the Sertoli cells by these chemicals.

The pattern of testicular alteration of germ cells and inhibition of spermatogenesis by the three chemicals with and without stress is suggestive of the pleiotropic effects of these exposures on nuclear DNA and cell division. Sertoli cells are commonly thought of as supportive cells within the seminiferous tubule providing a multitude of factors required for spermatogenesis (Russell et al., 1994; Sharpe, 1993). Furthermore, the loss of early germ cells can have profound effects on more mature germ cells in the spermatogenic process. Since the induction of germ-cell apoptosis in different animal models would disrupt spermatogenesis (Troiano et al., 1994), it is possible that germ-cell necrosis may contribute to the loss of cells seen in the present study. On the other hand, the lack of histologically necrotic cells or testicular inflammation and tissue scarring, which are common morphological signs of necrosis, negates the involvement of necrosis. The preferential loss of mature germ cells within the lumen of chemically treated or stressed and chemically treated testes sections (Figure 1, C and D) indicated a selective susceptibility of primary and secondary spermatocytes to a combination of PB, DEET, and permethrin-mediated cytotoxicity that was exacerbated by stress.

In the present study, monoclonal antibody recognizing ssDNA in apoptotic nuclei was used. This probe is considered to be a specific and sensitive cellular marker of apoptosis (Frankfurt et al., 1996). While TUNEL detects DNA fragmentation associated with late apoptosis, monoclonal antibody against ssDNA detects earlier stages of apoptosis and immunostained apoptotic cells in the absence of low-molecular-weight DNA fragmentation (Desjardins & MacManus, 1995; Frankfurt et al., 1996). The ssDNA detection technique makes use of the decreased stability of apoptotic DNA towards thermal denaturation, induced by the proteolysis of DNA-bound proteins during the execution of apoptosis (Frankfurt et al., 1996), as evidenced by the elimination of staining in sections reconstituted with histones before heating and the induction of staining by protease treatment (Frankfurt et al., 1997). The rationale behind the high specificity and sensitivity of this staining as compared

with TUNEL reflects the fact that protease activation is an early and probably universal signaling event in apoptosis (Martin & Green, 1995; Ashkenas & Werb, 1996).

In this study, treatment of animals with the combination of the three chemicals plus stress significantly increased the levels of nitrated proteins as detected with antibody against 3-nitrotyrosine. Furthermore, the result of 3-nitrotyrosine immunostaining closely paralleled the severity of tissue damage, and intense labeling was prominent in testes from rats treated with the combination of chemicals plus stress. Although the effect on the production of free radicals, particularly nitric oxide (NO), has not been previously studied, one might suggest that chemically induced testicular apoptosis is partly mediated by high levels of free radicals including NO. This suggestion is supported by the recent finding that endothelial nitric oxide synthase (NOS) activity was detected in human apoptotic germ cells (Zini et al., 1996). Although reactive oxygen species (ROS) and NO have been postulated to play an important role in the apoptosis of various cells, the precise mechanism leading to cell death remains to be fully elucidated (Rao, 2000). NOS has been localized to several cell types, including the rat testis and epididymis (Adams et al., 1992). NO was found to suppress the major regulatory aspects of testicular function, such as testosterone secretion and testicular interstitial fluid formation, suggesting an important role of NO in the control of testicular steroidogenesis, as well as regulating male fertility and sexual function (Adams et al., 1994; Punta et al., 1996). Moreover, combination of DEET and permethrin increased the urinary level of 8-hydroxy-2'-deoxyguanosine, a biomarker of oxidative DNA damage (Abu-Qare & Abou-Donia, 2000), and rat brain mitochondrial cytochrome c concentration (Abu-Qare & Abou-Donia, 2001b). Similarly, a combination of the three test compounds induced the urinary excretion of 3-nitrotyrosine (Abu-Qare et al., 2001). These findings might further confirm that testicular apoptosis is mediated by reactive oxygen and nitrogen species. Although the effect of free-radical scavengers (antioxidants) on chemically induced testicular apoptosis has not been examined in the current study, one might expect that a reduction in apoptosis and DNA damage would occur.

Pesticides and environmental chemicals may induce oxidative stress leading to generation of free radicals and alterations in antioxidants scavenging system (Agrawal et al., 1991; Almeida et al., 1997). Additionally, these chemicals may induce cytochrome P-450, and in the process of their metabolism, oxidants are generated that are capable of oxidizing a wide range of endogenous chemicals and xenobiotics (Hornsby, 1989). Recently, Abu-Qare and Abou-Donia (2001a) found that DEET alone and in combination with permethrin increased urinary excretion of 6- β -hydroxycortisol in rats, a marker of hepatic CYP3A induction.

Apoptosis is a well-characterized and common event in the adult rodent testes, where it is restricted mainly to spermatogonia (Allan et al., 1987). It thus appears that this normal process corresponds to a form of apoptosis that is not dependent on the presence of Bax, since this protein was found virtually

absent in control nonexposed rat testes. Bax is known to dimerize with Bcl2 and BclxL protein, and it has been shown that the balance between the expression of these apoptosis-protecting and apoptosis-inducing proteins is critical for cell survival or death (Korsmeyer, 1995). This is consistent with our findings that treatment of rats with a combination of test chemicals plus stress produced disturbances of this balance in the testes and increased induction of Bax gene expression, leading to accelerated apoptotic pathway.

The p53 protein, whose accumulation is induced by DNA damage and that participates in induction of apoptosis, was present in significant large amounts in testes from chemically treated plus stress rats, and decreased to very low levels in control nontreated rat germ cells. This protein is a positive regulator of Bax gene expression (Selvakumaran et al., 1994; Miyashita et al., 1994; Miyashita & Reed, 1995). Furthermore, it has been reported that p53 acts to regulate intracellular redox state and induces apoptosis by a pathway that is dependent on production of reactive oxygen species (Johnson et al., 1996). Additionally, ROS are known to be powerful inducers of p53 activity and play a key role in the execution of p53-dependent apoptosis (Shi et al., 1998; Pani et al., 2000). This suggests that the increased induction of Bax expression is perhaps in association with p53, since the p53 protein may act as a Bax promoter. Therefore, p53 may be instrumental in the apoptotic pathway associated with combined chemicals exposure. In conclusion, this study showed that the existence of apoptosis, coincident with a higher induction of Bax and p53 proteins.

Spermatogenesis is a complex and dynamic process that results in the continual production of spermatozoa in mammalian males. The Sertoli cells of the seminiferous epithelium are largely responsible for orchestrating the germ cells through sequential phases of mitosis, meiosis, and differentiation. The Sertoli cells accomplish this task by providing hormonal, nutritional, and physical support. An emerging theme from the present results is that apoptosis of testicular germ cells that occurs from chemical insults serves to reduce the germ-cell production. Chemicals that injure or disrupt the functions of Sertoli cells can effectively reduce their supportive capacity and result in an increased elimination of germ cells via apoptosis. These observations are likely to further fuel the widening interest in the mechanisms regulating apoptosis in the testes specifically, and the reproductive system in general, and may also explain the genitourinary disorders reported among some of the Persian Gulf War veterans.

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COMBINED EXPOSURE TO DEET (N,N-DIETHYL-*m*-TOLUAMIDE) AND PERMETHRIN: PHARMACOKINETICS AND TOXICOLOGICAL EFFECTS

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Permethrin and DEET are concurrently used for pests control inside homes, in public places, and in military shelters. Combined exposure to these compounds produced greater biochemical, behavioral, and metabolic alterations in animals compared to each individual compound. Concurrent application of DEET and permethrin induced urinary excretion of 3-nitrotyrosine and 8-hydroxy-2'-deoxyguanosine, markers of DNA damage and oxidative stress in rats, increased the release of rat brain mitochondrial cytochrome c, disrupted the blood-brain barrier (BBB) in rats, decreased m2 muscarinic acetylcholine receptor ligand binding density in rat brain, increased urinary excretion of 6 β -hydroxycortisol, a marker CYP3A4 induction, altered sensorimotor and locomotor activities in rats, and changed in vivo and in vitro metabolism and pharmacokinetic profiles of the individual compound. These findings show that more research is needed to examine adverse effects of the combined use of DEET and permethrin on other biochemical/physiological system(s) and to predict mechanistic pathways for these effects, particularly mechanism of action at cellular and molecular levels and alterations of genes transcription.

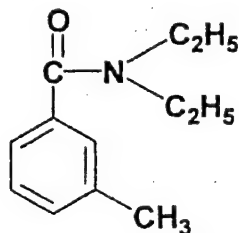
Concurrent exposure to chemicals has become of special concern, particularly when these compounds act via the same mechanism of action. Several studies reported on results of possible interactions between widely used chemicals such as insecticides, herbicides, industrial chemicals, or food additives.

Permethrin (3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl)methyl ester) (Figure 1) is a pyrethroid insecticide widely used in homes (WHO, 1990) and is effective against mites and head lice (Burgess et al., 1992; Fraser, 1994; Miller, 1989). The mechanism of action of permethrin is through interference with sodium channels, receptor-ionophore complexes, and neurotransmitters (WHO, 1990; Imamura et al., 2000). The oral LD₅₀ of permethrin in rats is between 3 and 5 g/kg (WHO, 1990). Garey and Wolff (1998)

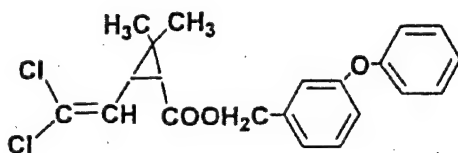
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DEET (*N,N*-diethyl-*m*-toluamide)



Permethrin

FIGURE 1. Chemical structures of DEET and permethrin.

reported that permethrin possessed estrogen and progesterone activities when incubated with human endometrial cancer and the T47D human breast cancer cell lines. Permethrin at a concentration of $100\mu\text{M}$ induced significant effects on proliferation of the MCF-7 human breast carcinoma cell line (Go et al., 1999). Further, permethrin altered genes expression in neurons of primary culture of mouse cerebellar granule cells (Imamura et al., 2000).

DEET (*N,N*-diethyl-*m*-toluamide) is applied as an insect repellent on the skin to act against mosquitoes, sand flies, ticks, and fleas (Robbins & Cherniak, 1986; Brown & Hebert, 1997). An insect repellent containing 10% or less DEET for children and no more than 30% DEET for adult was recommended to be used against mosquitoes carrying the West Nile virus (New York City Department of Health, 2000). The mode of action of DEET is less well understood. Poisoning symptoms included salivation, hyperexcitability, and sympathetic activation (Ray & Forshaw, 2000). Both chemicals were concurrently applied in homes and in public places (Burgess et al., 1992; U.S. EPA, 1998). Permethrin and DEET were used during the Gulf War to protect military personnel from insects and diseases (Abou-Donia et al., 1996; Young & Evans, 1998). Combined exposure to permethrin and DEET enhanced neurotoxicity in hens (Abou-Donia et al., 1996), increased mortality in rats (McCain et al., 1998), and caused behavioral alterations in rats (Hoy et al., 2000a, 2000b; Abou-Donia et al., 2001a, 2001b; Van Haaren et al., 2001). Reports implicated exposure to permethrin and DEET with Gulf War Illnesses (Abou-Donia et al., 1996; Haley and Kurt, 1997; Kurt, 1998; Shen, 1998; Wilson et al., 1998; Jamal, 1998; Haley et al., 1999). Recent studies showed that combined exposure to DEET and

permethrin induced the release of rat brain mitochondrial cytochrome c (Abu-Qare & Abou-Donia, 2001a), increased urinary excretion of 3-nitrotyrosine and 8-hydroxy-2-deoxyguanosine, biomarkers of oxidative stress in rats (Abu-Qare & Abou-Donia, 2000a; Abu-Qare et al., 2001b), increased urinary excretion of 6 β -hydroxycortisol, a marker of cytochrome P43A4 induction (Abu-Qare & Abou-Donia, 2001d), altered sensorimotor and locomotor activity in rats (Abou-Donia et al., 2001a), disrupted blood-brain barrier (BBB) permeability (Abou-Donia et al., 2001b), decreased m2 muscarinic ACh receptor ligand binding density in rat brain (Abdel-Rahman et al., 2001), and altered plasma area under curve (AUC) of the individual compound following combined dermal application in rats (Abu-Qare et al., 2001a).

PRODUCTION AND USE

Approximately 600 metric tons of permethrin is used every year worldwide (WHO, 1990). In total, 230 products containing DEET manufactured by about 70 different companies are currently registered with the U.S. Environmental Protection Agency (EPA), with an estimation that one-third of the U.S. population used DEET (U.S. EPA, 1998). Permethrin is most commonly used in electric insecticide vaporizers and mosquito coils that repel and kill mosquitoes and is effective against malaria, and DEET applied on the skin is effective against a wide range of blood-sucking arthropods (Coosemans & Guillet, 1999). The two compounds when tested in combination as a soap formulation provided 100% immediate protection against sand flies (Alexander et al., 1995). Another soap formulation of DEET and permethrin was very effective against eight mosquito species in India (Mani et al., 1991). Furthermore, DEET- and permethrin-containing clothing repellent offered the best overall protection against mites (Couch & Johnson, 1992). Volunteers wearing clothes impregnated with permethrin and with DEET formulation on exposed skin of face and arms were provided 91% protection against the natural population of tsetse flies in central Zambia (Sholdt et al., 1989). Under different climatic conditions, DEET and permethrin provided greater overall protection against *Aedes vigilax* in Thailand (Harbach et al., 1990), and against mosquitoes in Malaysia and Australia (Chiang et al., 1990; Yap, 1988; Frances, 1987). The most effective treatment against natural populations of mosquitoes in Pakistan was also achieved when both DEET and permethrin were used concurrently (Sholdt et al., 1988) (Table 1). Concurrent application of DEET and permethrin through the Department of Defense Insect Repellent System is widely used as a personal protective strategy to prevent insect-borne infectious diseases, where permethrin is usually applied on the battle dress uniform and DEET on the skin (Young & Evans, 1998; Evans et al., 1990).

BIOCHEMICAL EFFECTS

Additive or synergism interactions are likely between DEET and permethrin, where the effect would be equal to the sum of the effect of each chemical alone,

TABLE 1. Concurrent Use of DEET and Permethrin

Species	Formulation or place	Results	Reference
Mosquitoes, bites, insects	U.S. Army	Effective	Young and Evans (1998)
Sand flies	Soap formulation	100% Protection	Alexander et al. (1995)
Eight mosquito species	Soap formulation, India	Effective	Mani et al. (1991)
Lyme disease	Public places	Overall protection	Couch and Johnson (1992)
<i>Ae. vigilax</i>	Thailand	Protection	Harbach et al. (1990)
Mosquitoes	Malaysia	Efficient	Chiang et al. (1990)
Natural population of testse flies	Zambia	91% Protection	Sholdt et al. (1989)
Mosquitoes	Pakistan	Most effective protection	Sholdt et al. (1988)

or greater than would be expected from additive effects of each compound, respectively. Mechanisms of such interaction could be through (1) interference with absorption, distribution, and metabolism of each compound, (2) competition at the site of action, or (3) an alteration of physiological or biochemical or behavioral system (Abou-Donia, 1992).

Cholinergic System

Although published studies reported adverse effects following combined exposure to DEET and permethrin, issues such as dose, species, and method of application, as well as conditions resembling real-life situations, need further examination. Abou-Donia et al. (1996) reported that combinations of DEET and permethrin produced greater neurotoxicity in hens than that caused by any individual compound. Toxicological symptoms included gait disturbances, tremors, and mild to moderate microscopic changes in spinal cord and sciatic nerve of some hens (Table 2). In recent studies, combination of DEET and permethrin affected muscarinic and nicotinic receptors, and significantly inhibited cholinesterase enzymes in rats (Abou-Donia et al., 2001a; Abdel-Rahman et al., 2001). Abou-Donia et al. (2001a) reported that treatment of rats with 40 mg/kg of DEET and 0.13 mg/kg of permethrin for 45 d produced a significant decrease in acetylcholinesterase (AChE) activity in brainstem and midbrain, and significantly increased ligand binding for m2 muscarinic acetylcholine receptor (mAChR) in the cortex. Abdel-Rahman et al. (2001) applied daily doses of 40 mg/kg of DEET and 0.13 mg/kg of permethrin for 28 d in rats. They showed that a combined dose of DEET and permethrin significantly decreased brain AChE activity and significant neuronal cell death associated with a reduced microtubule-associated proteins (MAP-2) expression in the cerebral cortex and the hippocampus. In contrast, no significant changes were noticed following exposure to individual compounds.

Blood-Brain Barrier (BBB) and Blood-Testes Barrier (BTB)

Abou-Donia et al. (2001a, 2001b) reported that daily dermal combined doses of 4, 40, and 400 mg/kg of DEET and 0.013, 0.13, and 1.3 mg/kg of

TABLE 2. Biochemical Effects of Combined Administration of DEET and Permethrin

Effects	Dose	Species	References
Inhibition of AChE activity, enhanced neurotoxicity, increase in ligand binding for m2 muscarinic acetylcholine receptor (mAChR)	DEET 4–400 mg/kg, permethrin 0.13–1.3 mg/kg	Hens, rats	Abou-Donia et al. (1996, 2001a); Abdel-Rahman et al. (2001).
Disruption of blood–brain barrier (BBB) and blood–testes barrier (BTB) permeability	DEET 40 mg/kg, permethrin 0.13 mg/kg	Rats	Abou-Donia et al. (2001a, 2001b)
Induction of biomarkers of oxidative stress and apoptosis, e.g., 3-nitrotyrosine, 8-hydroxy-2-deoxyguanosine, cytochrome c	DEET 400 mg/kg, permethrin 1.3 mg/kg	Rats	Abu-Qare and Abou-Donia (2000a); Abu-Qare et al. (2001b, 2001c).
Increase urinary excretion of 6 β -hydroxycortisol	DEET 400 mg/kg, permethrin 1.3 mg/kg	Rats	Abu-Qare and Abou-Donia (2001d)
Caused significant neuronal cell death associated with reduced MAP-2 expression in the cerebral cortex and the hippocampus	DEET 40 mg/kg, permethrin 0.13 mg/kg	Rats	Abdel-Rahman et al. (2001)

permethrin for 60 d significantly decreased the BBB permeability in rat cortex, as assessed by injection of an iv dose of the quaternary ammonium compound [^3H]hexamethonium iodide. Blood–testes barrier (BTB) permeability was decreased by treatment of rats with daily dermal doses of 4, 40, and 400 mg/kg of DEET and 0.013, 0.13, and 1.3 mg/kg of permethrin for 60 days. This effect was more significant following combined exposure to DEET and permethrin compared to individual compound (Abou-Donia et al., 2001a, 2001b). Similarly, there was a reported decrease of (BBB) permeability (with focal perivascular accumulation of horseradish peroxidase [HRP] in both cerebrum and the brainstem) in rats exposed concurrently to daily doses of 40 mg/kg of DEET and 0.13 mg/kg of permethrin for 28 d (Abdel-Rahman et al., 2001).

Biomarkers of Apoptosis

Initiation of process of programmed cell death (apoptosis) is evidenced by biomarkers such as release of brain mitochondrial cytochrome c activity, activation of caspases, elevation of 8-hydroxy-2-deoxyguanosine levels, increased levels of 3-nitrotyrosine, and alterations of p53 gene expression (Abu-Qare & Abou-Donia, 2001a). These markers were examined following exposure to DEET and permethrin with an indication that significant effects were produced following combined exposure compared to an individual compound. Exposure of male rats to a single dermal dose of 400 mg/kg of DEET and 1.3 mg/kg of

permethrin significantly increased urinary concentrations of 8-hydroxy-2'-deoxyguanosine, a marker of DNA damage. The maximum increase appeared 48 h after the application of compounds (Abu-Qare & Abou-Donia, 2000a). In another study, Abu-Qare and Abou-Donia (2001b) reported that combined single dermal dose of 400 mg/kg of DEET and 1.3 mg/kg of permethrin significantly induced release of rat brain mitochondrial cytochrome c, a marker of programmed cell death (apoptosis). Abu-Qare et al. (2001c) measured the urinary levels of 3-nitrotyrosine in samples collected 2–72 h after a single dermal dose of 400 mg/kg of DEET and 1.3 mg/kg of permethrin, alone and in combination. The results showed that DEET and permethrin significantly increased concentrations of 3-nitrotyrosine compared to each individual compound. 3-Nitrotyrosine is believed to be formed through reaction of tyrosine with peroxynitrite. Levels of peroxynitrite increase as a result of interactions of oxidant radicals such as superoxide with nitric oxide following exposure to chemicals or in certain disease conditions (Roberts et al., 1998).

Cytochrome P-450 Induction

Cytochrome P-450 enzymes catalyze metabolism of a wide range of chemicals (Abou-Donia et al., 2001c). Effects of DEET and permethrin on cytochrome P-450 could have significant consequences on either the efficacy or the toxicity of common drugs that share metabolism. A study demonstrated that permethrin caused induction of cytochrome P-450 in rats (Koska et al., 1997). Recently, Heder et al. (2001) showed that permethrin induced cytochrome P-450 (CYP2B1) and (CYP3A1) mRNA in primary rat hepatocyte cultures. They reported that permethrin-stimulated gene expression occurred at the transcriptional level. Abu-Qare and Abou-Donia (2001d) measured the urinary excretion of 6 β -hydroxycortisol, a specific marker of CYP3A4 induction, 2–48 h following a combined single dermal dose of 400 mg/kg of DEET and 1.3 mg/kg of permethrin in rats. The results showed significant amounts of 6 β -hydroxycortisol were detected in urine samples starting 24 h following combined dose of DEET and permethrin. No such effect has been observed following application of each compound alone.

BEHAVIORAL ALTERATIONS

Behavioral alterations following combined dose of permethrin and DEET in humans are not well known. Several studies have been conducted examining animal (chicken and rats) behavior following exposure to each compound alone and in combination (Table 3). Abou-Donia et al. (2001b) reported that daily dermal combined doses of 4, 40, and 400 mg/kg of DEET and 0.013, 0.13, and 1.3 mg/kg of permethrin for 60 d produced a significant decline in sensorimotor performance in a dose- and time-dependent manner in rats that underwent a battery of functional behavior tests 30, 45, and 60 d after exposure. In another study, rats were treated with 40 mg/kg of DEET and 0.13 mg/kg of permethrin for 45 d. Their sensorimotor ability was assessed by a battery

TABLE 3. Behavioral Changes Following Combined Exposure to DEET and Permethrin

Symptoms	Dose	Species	References
Deficit in sensorimotor and locomotor function	DEET 40 mg/kg, permethrin 0.13 mg/kg	Male rats	Abou Donia et al. (2001a, 2001b)
Locomotor dysfunction	DEET 500 mg/kg, permethrin 500 mg/kg	Hens	Abou-Donia et al. (1996)
Lower speed rates, spent more time in the center zone, effect on thigmotaxis	DEET 50–500 mg/kg, permethrin 15–60 mg/kg.	Male rats	Hoy et al. (2000a)
Faster locomotion rate	Repeated daily dose for 7 d; DEET 100 mg/kg, permethrin 30 mg/kg	Male rats	Hoy et al. (2000b)
Disruption of well-established, schedule-controlled behavior	DEET 50–500 mg/kg, permethrin 15–60 mg/kg	Male and female rats	Van Haaren et al. (2001)

of behavioral tests that included beam-walk score, beak-walk time, inclined plane performance, and forepaw grip on d 30 and 45 following the treatment. The results showed that combined treatment with DEET and permethrin did have a significant effect on beam-walk score; the exposure to the two compounds resulted in a significant impairment in inclined plane testing on d 30 and 45 following treatment (Abou-Donia et al., 2001b). Hoy et al. (2000a) reported that combination of a single dose of DEET and permethrin significantly affected male rats speed and thigmotaxis where rats spent a significantly greater amount of time in the center zone than was expected based on averaging the single-drug values. In another study, Hoy et al. (2000b) reported that a repeated dose of DEET and permethrin resulted in a significantly faster locomotion rate in male rats, while this effect was not statistically significant in female rats. Furthermore, Van Haaren et al. (2001) showed that small dose of DEET and permethrin, alone and in combination disrupt well-established, schedule-controlled behavior in male and female rats. Even though such alterations of animal behavior following exposure to DEET and permethrin are well recognized, examining such effects in humans and providing mechanistic pathway and correlation with other effects need more study.

PHARMCOKINETIC INTERACTIONS

Interactions-based mixture risk assessment can be performed by simulating the change in the tissue dose of the toxic moiety of each mixture component during combined exposures and calculating the risk associated with each tissue dose estimate using tissue dose versus response curve for all components. The use of this mechanistic approach should facilitate the evaluation of the magnitude and relevance of chemical interactions in assessing the risks of

low-level human exposures to complex mixtures (Haddad & Krishnan, 1998). The metabolism of permethrin has been examined in rats (Anadon et al., 1991), where it was metabolized through hydrolysis and oxidation. Ray and Forshaw (2000) showed that carboxyesterase inhibitors can enhance permethrin toxicity in high-dose experimental studies. Abu-Qare and Abou-Donia (2001f) found that *iso*-OMPA (tetraisopropyl pyrophosphoramidate) a specific inhibitor of butyrylcholinesterase, significantly inhibited metabolism of permethrin following in vitro incubation with human liver microsomes. Recent studies showed that the esterase inhibitor organophosphorous compound DEF (S,S,S,-tri-n-butylphosphorotrithioate) and the cytochrome P-450 inhibitor piperonyl butoxide synergized the toxicity of the pyrethroid insecticide cypermethrin in larval and adult of *Helicoverpa zea*, *Sodoptera frugiperda*, and *Agrotis ipsilon* (Usmani & Knowles, 2001a, 2001b).

Disposition and metabolism of DEET has been studied in vivo, where it was rapidly absorbed, distributed, and eliminated following dermal application to human volunteers (Seliem et al., 1995; Blomquist & Thorsell, 1977), rats (Schoeing et al., 1996), mice (Blomquist & Thorsell, 1977), and cattle (Taylor et al., 1994) and after intravenous and topical application to beagle dogs (Qiu et al., 1997). DEET metabolism involved N-dealkylation, ring hydroxylation, and ring dealkylation following incubation with rat liver microsomes (Constantino & Iley, 1999; Taylor, 1986). Simultaneous exposure of DEET and permethrin to rodent and pig skin in vitro decreased permethrin absorption (Baynes et al., 1997). Hoy et al. (2000a) reported that concurrent administration of permethrin and DEET had no significant effect on the pyridostigmine bromide drug serum concentrations in male rats. Abu-Qare et al. (2001a) reported that following combined single dermal dose of 400 mg/kg of DEET and a dermal single dose of 1.3 mg/kg of permethrin in male rats, permethrin significantly increased systemic exposure of DEET in rat plasma as evidenced from its area under curve (AUC_{plasma}), while there was no significant effect on absorption. In another experiment, in vitro incubation of DEET and permethrin with human liver microsomes resulted in significant inhibition of rate of disappearance of either compound compared to incubation of individual ones (Abu-Qare & Abou-Donia, 2001e). These results provide evidence of the possible involvement of metabolism in toxic interactions between DEET and permethrin. It is possible that such interaction could result from a series of effects that ultimately lead to enhanced toxicity of the mixture.

METHODS OF ANALYSIS OF BOTH COMPOUNDS AND METABOLITES

Several analytical methods have been described in the literature for analysis of DEET and permethrin and their metabolites in biological matrices when applied as individual compounds. These methods mostly included using high-performance liquid chromatography (HPLC) (Qiu & Jun, 1996; Smallwood et al., 1992; Anadon et al., 1991; Garcia et al., 2001; Constantino & Iley, 1999;

Schoenig et al., 1996; Seliem et al., 1995), gas chromatography with different detectors, and gas chromatography-mass spectrometry (Heudorf & Angerer, 2001; Angerer & Ritter, 1997; Fraser et al., 1995). Limits of detection of these methods were within the range of nanograms per milliliter. Validated methods for the determination of DEET, permethrin, and their metabolites following combined application in rats were developed in our laboratories. Using HPLC with ultraviolet (UV) detection, DEET, permethrin, and selected metabolites were quantified in rats plasma and urine 24 h after a single dermal dose of 400 mg/kg of DEET and 1.3 mg/kg of permethrin. Limits of detection ranged between 20 and 100 ng/ml, while their recoveries were between 60 and 80% (Abu-Qare & Abou-Donia, 2000b, 2001c, 2001f).

SUMMARY

Concurrent application of permethrin and DEET is widely used in homes and in public health. It is also used as a personal protective strategy by the U.S. Department of Defense against insects. Several reports implicated combined exposure to permethrin and DEET to Gulf War Illnesses syndrome. Combined dermal exposure of both compounds enhanced neurotoxicity, increased lethality, caused oxidative stress in rats, induced cytochrome P-450 activity, disrupted blood-brain barrier (BBB) and blood-testes barrier (BTB) permeability, induced behavioral and biochemical changes, and interfered with pharmacokinetic profiles in vivo and in vitro.

The wide use of either a single formulation of DEET and permethrin or their concurrent use is a good reason for further examination of possible interactions under real-life conditions. Their possible interactions at the cellular and molecular levels as well as their interference with genes transcription need further study. The nature and magnitude of the impacts following chronic or sub-chronic exposure need further investigation. More research is needed examining developmental effects and the susceptibility of children and pregnant women to such combined exposure, since both chemicals are used in homes and public places.

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Co-exposure to pyridostigmine bromide, DEET, and/or permethrin causes sensorimotor deficit and alterations in brain acetylcholinesterase activity

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Abstract

Military personnel deployed in the Persian Gulf War (PGW) were exposed to a combination of chemicals, including pyridostigmine bromide (PB), DEET, and permethrin. We investigated the dose–response effects of these chemicals, alone or in combination, on the sensorimotor performance and cholinergic system of male Sprague–Dawley rats. Animals were treated with a daily dermal dose of DEET and/or permethrin for 60 days and/or PB (gavage) during the last 15 days. Neurobehavioral performance was assessed on day 60 following the beginning of the treatment with DEET and permethrin. The rats were sacrificed 24 h after the last treatment for biochemical evaluations. PB alone, or in combination with DEET, or DEET and permethrin resulted in deficits in beam-walk score and longer beam-walk times compared to controls. PB alone, or in combination with DEET, permethrin, or DEET and permethrin caused impairment in incline plane performance and forepaw grip strength. PB alone at all doses slightly inhibited plasma butyrylcholinesterase activity, whereas combination of PB with DEET or permethrin increased its activity. Brainstem acetylcholinesterase (AChE) activity significantly increased following treatment with combinations of either DEET or permethrin at all doses, whereas the cerebellum showed a significant increase in AChE activity following treatment with a combination of PB/DEET/permethrin. Co-exposure to PB, DEET, and permethrin resulted in significant inhibition in AChE in midbrain. PB alone or in combination with DEET and permethrin at all doses increased ligand binding for m2 muscarinic acetylcholine receptor in the cortex. In addition, PB and DEET together or a combination of PB, DEET, and permethrin significantly increased ligand binding for nicotinic acetylcholine receptor. These results suggest that exposure to various doses of PB, alone and in combination with DEET and permethrin, leads to sensorimotor deficits and differential alterations of the cholinergic system in the CNS.

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Keywords: Persian Gulf War; Sensorimotor; Pyridostigmine bromide; DEET; Permethrin; Combined exposure; Acetylcholinesterase; Butyrylcholinesterase; m2 muscarinic acetylcholine receptor; Nicotinic acetylcholine receptor; Brain

1. Introduction

Military personnel deployed in the Persian Gulf War (PGW) were exposed to a unique combination of chemical, biological, and psychological environments. Since their return from the PGW, many of personnel have had symptoms including chronic fatigue, muscle and joint pain, ataxia, rash, headache, difficulty concentrating, forgetful-

ness, and irritability (Institute of Medicine, 1995; Haley et al., 1997a). Combined chemical exposures included a variety of organophosphate compounds, the insect repellent *N,N*-diethyl-*m*-toluamide (DEET), and the pyrethroid insecticide, permethrin (Institute of Medicine, 1995). Additionally, these veterans were given a course of twenty-one 30-mg tablets of pyridostigmine bromide (PB) as prophylaxis against organophosphate nerve agents (Persian Gulf Veterans Coordinating Board, 1995).

PB is considered to be relatively safe to humans at the doses given. It is a quaternary dimethyl carbamate that has been used primarily as a treatment for myasthenia gravis at

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higher doses (Breyer-Pfaff et al., 1985, 1990) than that given to PGW veterans (Keeler et al., 1991; Golomb, 1999; Kant et al., 2001). It reversibly inhibits acetylcholinesterase (AChE) in the peripheral nervous system (PNS), thus limiting irreversible inhibition of the enzyme by nerve agents (Blick et al., 1991). AChE activity is restored following spontaneous decarbamylation resulting in near-normal neuromuscular and autonomic functions (Blick et al., 1991). Toxic symptoms associated with PB overdose result from overstimulation of nicotinic and muscarinic receptors in the PNS, resulting in exaggerated cholinergic effects, such as muscle fasciculations, cramps, weakness, muscle twitching, tremor, respiratory difficulty, gastrointestinal tract disturbances, and paralysis (Abou-Donia et al., 1996). Central nervous system (CNS) effects of PB are not expected unless blood–brain barrier (BBB) permeability is compromised Birtley et al., 1966. CNS effects of PB are variable depending on the species and experimental model (mice, Friedman et al., 1996; Grauer et al., 2000; rat, Sinton et al., 2000; Li et al., 2000; Kant et al., 2001; guinea pigs, Lallemand et al., 1998, 2001).

The insect repellent *N,N*-diethyl-*m*-toluamide (DEET) has been extensively used by humans since its introduction. DEET is commonly used as a repellent against mosquitoes, flies, ticks, and other insects (Robbins and Cherniack, 1986; McConnell et al., 1986; Pollack et al., 2002; Fradin and Day, 2002). Extensive and repeated topical DEET applications can be toxic in humans (Gryboski et al., 1961; Roland et al., 1985; Edwards and Johnson, 1987). The symptoms associated with DEET poisoning include tremors, restlessness, difficulty with speech, seizures, impairment of cognitive function, coma, and death (McConnell et al., 1986). Although the exact mechanisms of DEET toxicity are not known, exposure to extremely high levels of DEET produces demyelination and spongiform myelinopathy in the rat (Verschoyle et al., 1992). DEET efficiently crosses the dermal barrier (Windheuser et al., 1982; Hussain and Ritschel, 1988) and may concentrate in fat (Blomquist and Thorsell, 1977; Snodgrass et al., 1982).

Permethrin [3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid (3-phenoxyphenyl) methyl ester], a type I synthetic pyrethroid insecticide, exists in four different stereoisomers (Casida et al., 1983). It provides insecticidal activity for several weeks following a single application and is used to control fleas, flies, mites, and cockroaches. Permethrin causes modification of sodium channels leading to prolonged depolarization and repetitive discharges in presynaptic nerve fibers after a single stimulus (Narahashi, 1985). This repetitive nerve action is associated with tremor, hyperactivity, ataxia, convulsions, and in some cases paralysis.

We previously reported that subchronic exposure to 0.1 ×, 1 ×, and 10 × doses of DEET (4, 40, and 400 mg/kg, respectively, daily dermal) and permethrin (0.013, 0.13, and 1.3 mg/kg, respectively, daily dermal), either alone or in combination, caused sensorimotor deficits (Abou-Donia et

al., 2001a). The 1 × dose of each chemical was based on the estimation by the Department of Defense for the exposure to these chemicals to the deployed military personnel in the PGW in 1991. The present study was carried out to evaluate the interactive effects on sensorimotor performance and the cholinergic system following exposure to various doses of PB, alone and in combination of DEET and permethrin.

2. Materials and methods

2.1. Chemicals

Technical-grade (>93.6%) permethrin 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid (3-phenoxyphenyl) methyl ester was obtained from Roussel Uclaf (Pasadena, TX). DEET (99.7% *N,N*-diethyl-*m*-toluamide), PB (≥ 99%, 3-dimethylamino carbonyl oxy-*N*-methyl pyridinium bromide), acetylthiocholine iodide, butyrylthiocholine iodide, atropine, and nicotine were purchased from Sigma (St. Louis, MO). 5,5'-Dithio-bis-2-nitrobenzoic acid (DTNB) was purchased from Aldrich. The inhibitor, 1,5-bis-(*N*-allyl-*N,N*-dimethyl-4-ammonium phenyl) pentan-3-one dibromide (BW284C51) was obtained from Sigma. [³H]AF-DX 384, specific activity 106 μCi/mmol and [³H]cytisine, specific activity 32 nCi/pmol were purchased from New England Nuclear (Boston, MA). All other chemicals and reagents were of highest purity available from commercial sources.

2.2. Animals

Male Sprague–Dawley rats weighing 200–250 g were obtained from Zivic-Miller Laboratories (Allison Park, PA) and housed at Duke University Medical Center vivarium. The animals were randomly assigned to control and treatment groups and housed at 21–23 °C with a 12-h light/dark cycle. They were supplied with food and water ad libitum. The rats were allowed to adjust to their environment before starting the treatment. All the experimental studies on the rats were carried out strictly according to the U.S. Army and Duke University Institutional Animal Care and Use Committee.

2.3. Treatment

The rats were randomly divided into following groups (*n* = 5 per group):

1. Controls: received 70% ethanol dermally and water by gavage orally each day.
2. PB alone: PB (0.1 ×, 1 ×, or 10 ×; 0.13, 1.3, or 13 mg/kg, respectively) was given daily by gavage for the last 15 days of the experiment, from days 46 to 60.
3. PB + DEET: DEET (0.1 ×, 1 ×, or 10 ×; 4, 40, or 400 mg/kg, respectively, dermal) was given daily for 60 days.

In addition, during the last 15 days, the rats were treated orally with PB ($0.1 \times$, $1 \times$, or $10 \times$; 0.13, 1.3, or 13 mg/kg, respectively) daily by gavage.

4. PB+permethrin: Permethrin ($0.1 \times$, $1 \times$, or $10 \times$; 0.013, 0.13, or 1.3 mg/kg, respectively, dermal) was given daily for 60 days. In addition, during the last 15 days, the rats were treated orally with PB ($0.1 \times$, $1 \times$, or $10 \times$; 0.13, 1.3, or 13 mg/kg, respectively) daily by gavage.
5. PB+DEET+permethrin: DEET ($0.1 \times$, $1 \times$, or $10 \times$; 4, 40, or 400 mg/kg, respectively, dermal) and permethrin ($0.1 \times$, $1 \times$, or $10 \times$; 0.013, 0.13, or 1.3 mg/kg, respectively, dermal) were given daily for 60 days. In addition, during the last 15 days, the rats were treated orally with PB ($0.1 \times$, $1 \times$, or $10 \times$; 0.13, 1.3, or 13 mg/kg, respectively) daily by gavage.

DEET and permethrin were applied directly to the pre-clipped skin on 1 in.^2 of the back of the neck. The chemicals were applied to give the desired concentration of test compounds in 0.1 ml of vehicle. DEET was given in doses of 4, 40, or 400 mg/kg ($0.1 \times$, $1 \times$, and $10 \times$ dose, respectively) in ethanol with or without permethrin (0.013, 0.13, or 1.3 mg/kg; $0.1 \times$, $1 \times$, and $10 \times$ dose, respectively). Controls received an equal volume of the vehicle. The treatment with DEET and permethrin was carried out for 60 days, whereas the treatment with PB was during the last 15 days of the experiment.

The $1 \times$ dose of PB, DEET, and permethrin corresponds to an estimate of the military personnel's exposure to these agents during the PGW as provided by the Department of Defense. Animals were sacrificed 24 h after the treatment with the last dose.

2.4. Behavioral studies

A comprehensive battery of standardized tests was employed on day 60 following the beginning of the treatment with DEET or permethrin. These behavioral tests were designed to measure sensorimotor reflexes, motor strength, and coordinated gait (Bederson et al., 1986; Markgraf et al., 1992; Goldstein, 1993, 1995). All behavioral testing was performed by a trained observer blind to the animal's treatment status, and was carried out in a soundproof room with subdued lighting (less than 10.76 lumens/m^2 , ambient light). Rats were handled for 2 min daily for 5 days during the week prior to behavioral testing. The behavioral evaluations commenced 2 h following the last treatment with the last dose. All behavioral tests were carried out in each group in the same order to maintain the time elapsed after the last treatment.

2.4.1. Beam walking and beam score

2.4.1.1. Description. The testing apparatus is a $2.5 \times 122 \text{ cm}$ wooden beam elevated 75.5 cm above the floor with

wooden supports. A $20 \times 25 \times 24 \text{ cm}$ goal box with a 9.5 cm opening is located at one end of the beam. A switch-activated source of bright light (75 W Tungsten bulb) and white noise are located at the start–end of the beam and serve as avoidance stimuli. The rats were first trained with a series of three approximate trials (Goldstein, 1993, 1995).

2.4.1.2. Scoring. For the testing trials, rats were placed at the start–end of the beam, near the sources of light and noise. Both the latency until the animal's nose entered the goal box (up to 90 s) and the use of the hind paw to aid locomotion was recorded on a seven-point scale: 7 = normal performance, 1 = unable to place hind paw onto the horizontal surface of the beam. Rats that fell off the beam were assigned latencies of 90 s.

2.4.2. Incline plane

The rats were placed on a platform that was elevated at increasing angles. The angle at which the animal fell off the platform was recorded. The results of two trials were averaged at each time point.

2.4.3. Grip time

Rats forepaw grip strength was assessed by having the animal's forepaw grip a 5-mm diameter wood dowel. Time to release their grip was recorded in seconds.

2.4.4. Statistical analysis

Data for beam-walk time and grip time were analyzed with two-way (drug group and dose) ANOVA with post hoc planned contrasts between each group and controls. Because the data were not normally distributed, data for beam-walk score and incline plane performance were first analyzed with the Kruskal–Wallis test. The Mann–Whitney *U* test (corrected for multiple comparisons) was then used to compare each group with controls. Two-tailed $P < .05$ was considered significant.

2.5. Tissue retrieval for biochemical evaluations

Twenty-four hours after the treatment with the last dose, the rats were anesthetized with intraperitoneal injection of ketamine/xylazine (100 mg/kg ketamine and 15 mg/kg xylazine). Blood was drawn by cardiac puncture and brains were dissected out. The brains were washed thoroughly in ice-cold normal saline and brain regions were dissected on ice and frozen immediately in liquid nitrogen. Plasma was separated and frozen at -80°C until enzyme studies were done.

2.5.1. Acetylcholinesterase and butyrylcholinesterase

Brain AChE and plasma cholinesterase (BChE) activities were measured by the Ellman assay (Ellman et al., 1961). For AChE assays, dissected brain regions were homogenized in Ellman buffer, centrifuged for 5 min at $5000 \times g$, and the resulting supernatant used for AChE analysis. AChE activity

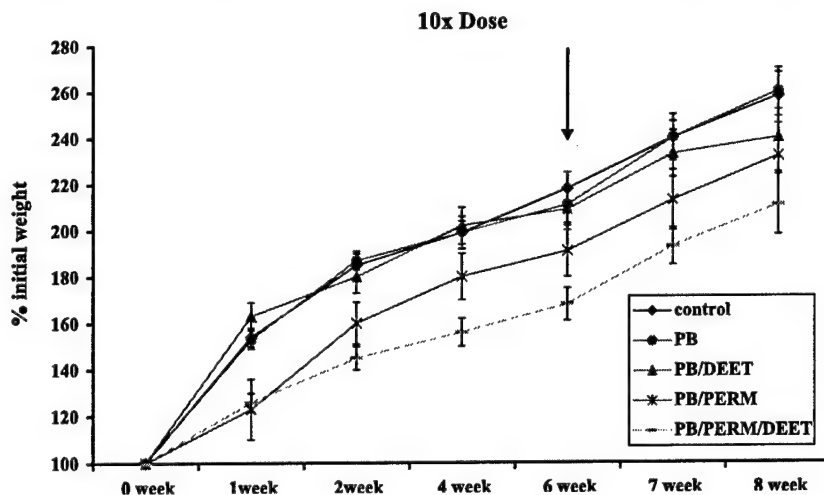


Fig. 1. Body weight gain following treatment with $10 \times$ dose PB, alone or in combination with $10 \times$ dose of DEET and permethrin. The rats were treated with PB ($0.1 \times$, $1 \times$, or $10 \times$; 0.13, 1.3, or 13 mg/kg, respectively, daily by gavage), PB+DEET ($0.1 \times$, $1 \times$, or $10 \times$; 4, 40, or 400 mg/kg, respectively, dermal, daily for 60 days), PB+permethrin ($0.1 \times$, $1 \times$, or $10 \times$; 0.013, 0.13, or 1.3 mg/kg, respectively, dermal, daily for 60 days), or PB+DEET+permethrin as above. The rats in the PB-alone group or in the combination with PB groups were treated with PB during the last 15 days orally with PB ($0.1 \times$, $1 \times$, or $10 \times$; 0.13, 1.3, or 13 mg/kg, respectively, daily by gavage) as described in Materials and Methods. Weight of the rats was recorded and the data are plotted as mean \pm S.E. of weight gain as percent initial weight. Arrow represents the beginning of the oral treatment with PB by gavage.

was measured using acetylthiocholine as substrate in a Molecular Devices UV Max Kinetic Microplate Reader at 412 nm as described by Abou-Donia et al. (2001b). The enzyme activity is expressed as nanomoles of substrate hydrolyzed per minute per milligram of protein. Protein concentrations in tissue samples and plasma were determined by the method of Smith et al. (1985).

2.5.2. Muscarinic acetylcholine receptor binding

For the assay of the ligand binding for m2 muscarinic acetylcholine, the tissue was homogenized in 10 mM phosphate buffer, pH 7.4, and centrifuged at $40,000 \times g$ for 10 min, and the membranes were suspended in the same buffer at a protein concentration of 1.25–2.5 mg/ml as described by Huff et al. (1994), and the ligand binding was carried out according to Slotkin et al. (1999). The m2 muscarinic acetylcholine binding was carried out by using m2 muscarinic acetylcholine-specific ligand, [3 H]AF-DX 384 at room temperature for 60 min. Nonspecific binding was carried out in the presence of 2.2 μ M atropine sulfate. Ligand-bound membranes were trapped on glass filters presoaked with 0.1% polyethylenimine using a rapid vacuum filtration system.

2.5.3. Nicotinic acetylcholine receptor binding

[3 H]Cytisine was used as specific ligand for nicotinic acetylcholine according to the method described by Slotkin et al. (1999). Tissues were homogenized by Polytron in 50 mM Tris-HCl pH 7.4 containing 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , and 2.5 mM MgCl_2 . The membranes were sedimented by centrifuging at $40,000 \times g$ for 10 min at 4°C . The resulting pellet was resuspended in the same buffer using Teflon pestle glass homogenizer in a volume to give 1.5–2 mg/ml protein. An aliquot of membrane preparation containing $\sim 200 \mu\text{g}$ protein was used to carry out the incubation with 1 nM [3 H]cytisine at 4°C for 75 min. Nonspecific binding was carried out in the presence of 2 μM nicotine bitartrate. Ligand-bound membranes were trapped on membrane filters using rapid vacuum filtration system.

2.5.4. Statistics

The results were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. Data for body weight gain were analyzed by two-way ANOVA for the treatment effects and time interaction. $P < .05$ was considered significant.

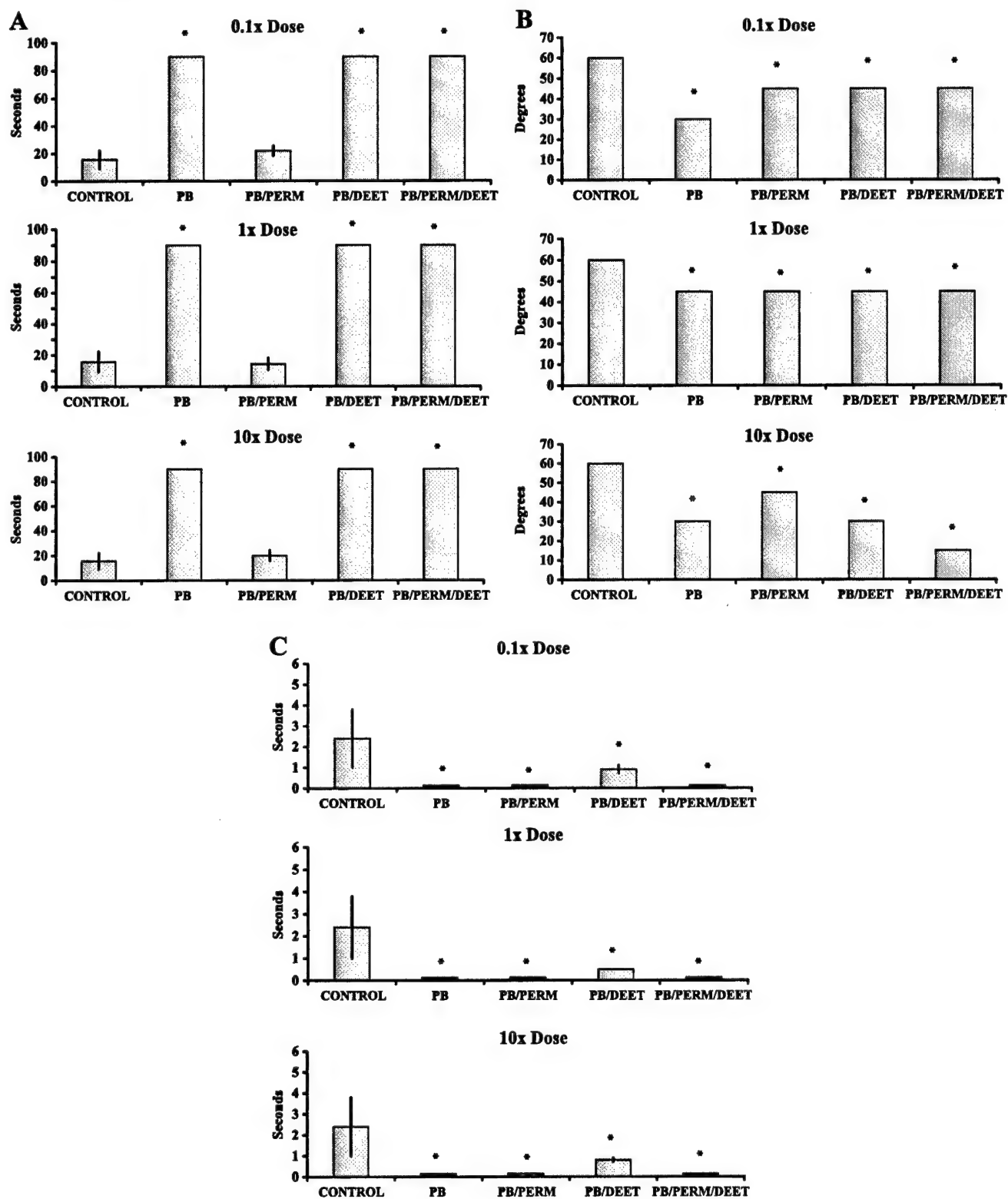
Fig. 2. Effect of treatment with $0.1 \times$, $1 \times$, or $10 \times$ dose of PB alone or in combination with DEET and permethrin on sensorimotor performance: The rats were treated with PB ($0.1 \times$, $1 \times$, or $10 \times$; 0.13, 1.3, or 13 mg/kg, respectively, daily by gavage), PB+DEET ($0.1 \times$, $1 \times$, or $10 \times$; 4, 40, or 400 mg/kg, respectively, dermal, daily for 60 days), PB+permethrin ($0.1 \times$, $1 \times$, or $10 \times$; 0.013, 0.13, or 1.3 mg/kg, respectively, dermal, daily for 60 days), or PB+DEET+permethrin as above. The rats in the PB-alone group or in the combination with PB groups were treated with PB during the last 15 days orally with PB ($0.1 \times$, $1 \times$, or $10 \times$; 0.13, 1.3, or 13 mg/kg, respectively, daily by gavage) as described in Materials and Methods. The rats were tested 2 h after the last treatment. Beam-walk time (A), incline plane (B), and forepaw grip time (C). Top panel: Data were obtained following exposure with $0.1 \times$ dose of PB alone or in combination with DEET and permethrin. Middle panel: Data were obtained following exposure with $1 \times$ dose of PB alone or in combination with DEET and permethrin. Bottom panel: Data were obtained following exposure with $10 \times$ dose of PB alone or in combination with DEET and permethrin. Data in (A) and (C) are presented as mean \pm S.E. * Statistically significant ($P < .05$) as compared with controls, corrected for multiple comparisons. Data in (B) are medians, interquartile range=0 for each condition. For incline plane, all treatment groups were significant when compared with Mann-Whitney U , corrected for multiple comparisons.

3. Results

3.1. General health and clinical condition

Animals showed no overt clinical signs of toxicity observed throughout the study. There were no significant

effects on the body weight gain in the rats treated with $0.1 \times$ or $1 \times$ dose. The rats treated with $10 \times$ dose of PB and permethrin and that of $10 \times$ dose of PB/DEET/permethrin gained less weight than controls or those treated with PB and DEET (Fig. 1). Two-way ANOVA for the treatment effects and time interaction did not show any significant effects.



3.2. Effect of treatment with 0.1×, 1×, or 10× dose of PB alone or in combination with DEET and permethrin on sensorimotor performance

Fig. 2 shows the results of the behavioral tests after 60 days exposure to 0.1×, 1×, or 10× dose of DEET and permethrin in combination with PB. PB treatment was carried out during the last 15 days of the experiment.

3.2.1. Beam-walk score

There was a significant difference among the groups at each dosing level (0.1× dose, Kruskal–Wallis $H=29$, $P<.0001$; 1× dose, Kruskal–Wallis $H=28$, $P<.0001$; 10× dose, Kruskal–Wallis $H=25$, $P<.001$). Except for

the combination of PB and permethrin (median score=7, interquartile range=0 for each dose), each group (PB, PB+permethrin, PB+permethrin+DEET) significantly differed from controls (median score 7, interquartile range=0) at each dosing level (median score 1, interquartile range=0, for each group at each dose level; Mann–Whitney U corrected for multiple comparisons, $P<.002$ for each comparison).

3.2.2. Beam-walk time

Two-way ANOVA showed a significant effect of drug group [$F(3,52)=1572$, $P<.00001$] but no effect of dose [$F(2,52)=2.02$, $P=.14$] and no Group × Dose interaction [$F(6,52)=2.05$, $P=.08$]. Fig. 2A shows the planned com-

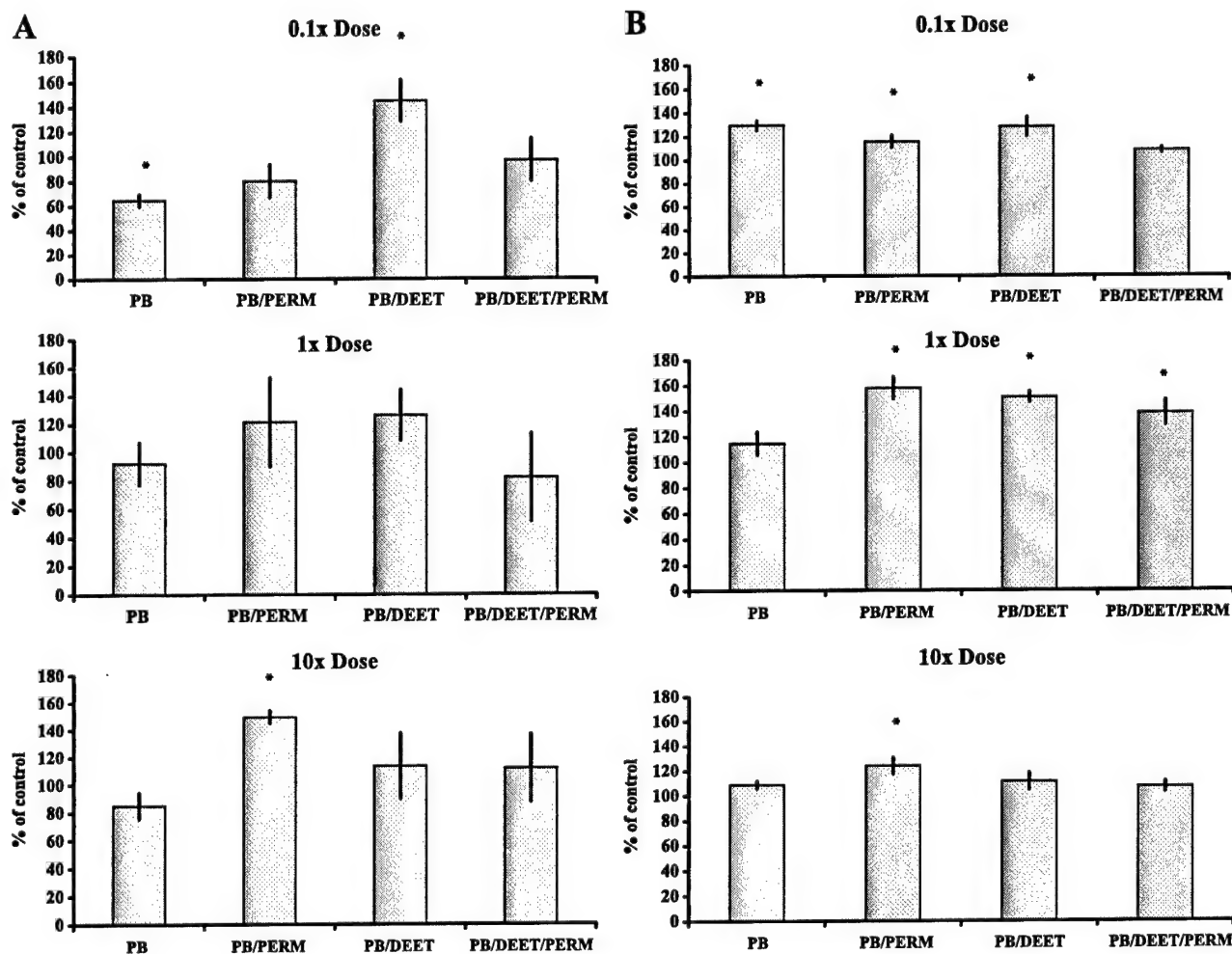


Fig. 3. Effect of treatment with 0.1×, 1×, or 10× dose of PB alone or in combination with DEET and permethrin on plasma butyrylcholinesterase and brain regional AChE activity. The rats were treated with PB (0.1×, 1×, or 10×; 0.13, 1.3, or 13 mg/kg, respectively, daily by gavage), PB+DEET (0.1×, 1×, or 10×; 4, 40, or 400 mg/kg, respectively, dermal, daily for 60 days), PB+permethrin (0.1×, 1×, or 10×; 0.013, 0.13, or 1.3 mg/kg, respectively, dermal, daily for 60 days), or PB+DEET+permethrin as above. The rats in the PB-alone group or in the combination with PB groups were treated with PB during the last 15 days orally with PB (0.1×, 1×, or 10×; 0.13, 1.3, or 13 mg/kg, respectively, daily by gavage) as described in Materials and Methods. Twenty-four hours after the last treatment, the animals were sacrificed, and plasma and brain regions were evaluated for the enzyme activities. (A) Plasma and (B) brainstem. Top panel: Data were obtained following exposure with 0.1× dose of PB alone or in combination with DEET and permethrin. Middle panel: Data were obtained following exposure with 1× dose of PB alone or in combination with DEET and permethrin. Bottom panel: Data were obtained following exposure with 10× dose of PB alone or in combination with DEET and permethrin. Enzyme activity (nmol/min/mg protein) in the control animals: plasma, 0.04 ± 0.001 ; brainstem, 1.29 ± 0.09 . Data are presented as mean ± S.E. (percent of control). * Statistically significant ($P<.05$).

parison contrasts for each treatment group at each dose level vs. control [$F(1,52)$]. All comparisons were significant except for the combination of PB and permethrin at the $1 \times$ dose.

3.2.3. Incline plane

There was a significant difference among the groups at each dosing level ($0.1 \times$ dose, Kruskal–Wallis $H=29$, $P<.0001$; $1 \times$ dose, Kruskal–Wallis $H=28$, $P<.0001$; $10 \times$ dose, Kruskal–Wallis $H=25$, $P=.001$). Fig. 2B shows the median angles for each treatment group at each dose level vs. control. Each group significantly differed from controls at each dosing level (Mann–Whitney U , corrected for multiple comparisons, $P<.002$ for each comparison).

3.2.4. Forepaw grip

Two-way ANOVA showed a significant effect of drug group [$F(3,52)=5.34$, $P<.003$] but no effect of dose [$F(2,52)=0.14$, $P=.87$] and no Group \times Dose interaction [$F(6,52)=0.15$, $P=.99$]. Fig. 2C shows the planned comparison contrasts for each treatment group at each dose level vs. control [$F(1,52)$]. Each group significantly differed from controls at each dosing level.

3.3. Effect of treatment with $0.1 \times$, $1 \times$, or $10 \times$ dose of PB alone or in combination with DEET and permethrin on plasma BChE and brain regional ache activity

Fig. 3A shows the effects of treatment with $0.1 \times$, $1 \times$, or $10 \times$ dose of PB alone or in combination with DEET and permethrin on plasma BChE activity at three different doses (top panel, $0.1 \times$ dose; middle panel, $1 \times$ dose; bottom panel, $10 \times$ dose). Data on the effects of single chemical treatment are presented in Fig. 3 (top panel). Treatment with $0.1 \times$ PB alone produced significant inhibition ($\sim 65\%$ of controls) of plasma BChE activity. Treatment with PB and DEET at $0.1 \times$ dose produced a significant increase ($\sim 145\%$ of control) whereas no significant changes were observed in combination with PB and permethrin or a combination with PB, DEET, and permethrin. At $10 \times$ dose (bottom panel), a combination of permethrin and PB caused a significant increase ($\sim 149\%$ of control) in plasma BChE activity.

Fig. 3B shows the effects of treatment with $0.1 \times$, $1 \times$, or $10 \times$ dose of PB alone or in combination with DEET and permethrin on AChE activity in the brainstem (top panel, $0.1 \times$ dose; middle panel, $1 \times$ dose; bottom panel, $10 \times$ dose). There was a significant increase in the brainstem AChE activity at $0.1 \times$ dose of PB alone or in combination with DEET or permethrin (upper panel). At $1 \times$ dose, a combination of PB/DEET or PB/permethrin or PB/DEET/permethrin resulted in a significant increase (~ 138 – 150% of control) in AChE activity (middle panel), whereas at $10 \times$ dose only the combination of PB/permethrin caused a significant increase ($\sim 123\%$ of control) (lower panel). Cerebellum AChE activity showed a significant increase

at $1 \times$ dose following treatment with a combination of PB/DEET/permethrin (data not shown) or $10 \times$ dose with a combination of PB/permethrin (data not shown). A combination of PB/DEET/permethrin at $10 \times$ dose resulted in a significant decrease ($\sim 88\%$ of control) in cerebellum AChE activity (data not shown).

3.4. Effect of treatment with $0.1 \times$, $1 \times$, or $10 \times$ dose of PB alone or in combination with DEET and permethrin on brain $m2$ muscarinic and nicotinic acetylcholine receptor ligand binding

To evaluate the effects of treatment with $0.1 \times$, $1 \times$, or $10 \times$ dose of PB, alone and in combination with DEET and permethrin, on muscarinic and nicotinic acetylcholine receptor ligand binding studies were carried out with membrane preparations. For $m2$ muscarinic acetylcholine receptors, $m2$ -specific ligand, [3H]AFDX was used. The data on $1 \times$ dose are presented in Fig. 4. PB treatment alone or in combination with DEET or a combination of PB/DEET/permethrin produced a significant increase in ligand binding density in the cortex (~ 130 – 145% of control). Similarly, at $0.1 \times$ and $10 \times$ dose, treatment with PB alone or in combination with DEET or permethrin or DEET and permethrin resulted in significant increase in the ligand binding (data not shown); however, there was no difference among the doses.

Ligand binding for nicotinic acetylcholine receptors using [3H]cytisine was carried out in the cortex membranes

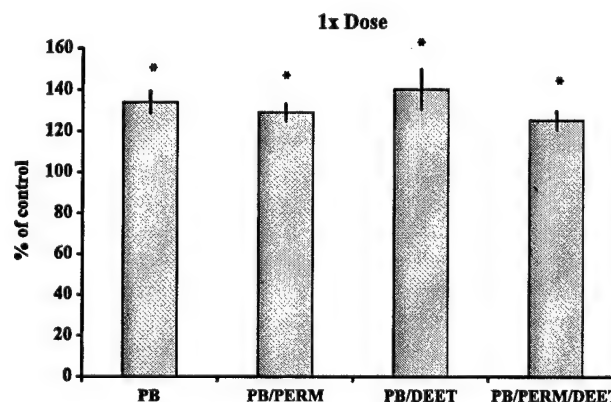


Fig. 4. Effect of treatment with $1 \times$ dose of PB alone or in combination with DEET and permethrin on $m2$ muscarinic acetylcholine receptor ligand binding in the cortex. The rats were treated with PB ($1 \times$; 1.3 mg/kg, daily by gavage), PB+DEET ($1 \times$; 40 mg/kg, dermal, daily for 60 days), PB+permethrin ($1 \times$; 0.13 mg/kg, respectively, dermal, daily for 60 days), or PB+DEET+permethrin as above. The rats in the PB-alone group or in the combination with PB groups were treated with PB during the last 15 days orally with PB ($1 \times$; 1.3 mg/kg, daily by gavage) as described in Materials and Methods. Twenty-four hours after the last treatment, the animals were sacrificed. The details of membrane preparation and [3H]AFDX 384 ligand binding assay are elaborated in Materials and Methods. Ligand binding in control membranes: 51 ± 4.1 fmol/mg protein. Data are presented as mean \pm S.E. (percent of control). * Statistically significant ($P<.05$).

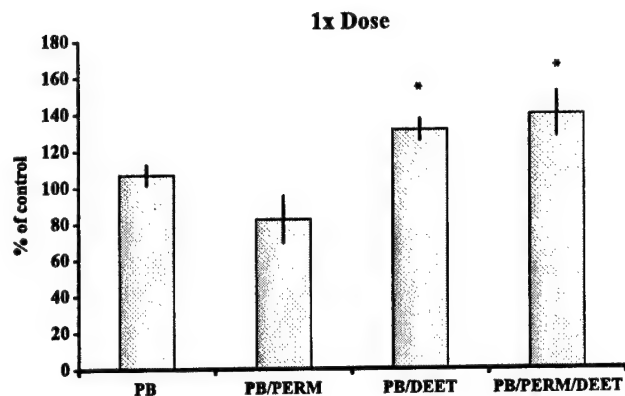


Fig. 5. Effect of treatment with 1 \times dose of PB alone or in combination with DEET and permethrin on nicotinic acetylcholine receptor ligand binding in cortex. The rats were treated with PB (1 \times ; 1.3 mg/kg, daily by gavage), PB+DEET (1 \times ; 40 mg/kg, dermal, daily for 60 days), PB+permethrin (1 \times ; 0.13 mg/kg, respectively, dermal, daily for 60 days), or PB+DEET+permethrin as above. The rats in the PB-alone group or in the combination with PB groups were treated with PB during the last 15 days orally with PB (1 \times ; 1.3 mg/kg, daily by gavage) as described in Materials and Methods. Twenty-four hours after the last treatment, the animals were sacrificed. The details of membrane preparation and [3 H]cytisine ligand binding assay are elaborated in Materials and Methods. Ligand binding in control membranes: 23.9 \pm 4.1 fmol/mg protein. Data are presented as mean \pm S.E. (percent of control). * Statistically significant ($P < .05$).

prepared from the animals treated with 0.1 \times , 1 \times , and 10 \times dose of PB, alone and in combination with DEET and permethrin. The data are presented in Fig. 5 for 1 \times dose only. Treatment with PB/DEET or PB/DEET/permethrin caused a significant increase in the ligand binding (\sim 130% of control). Treatment with PB alone at any dose did not produce any significant change in ligand binding. There was no dose response.

4. Discussion

In the present study, we evaluated the neurotoxic response following exposure with 0.1 \times , 1 \times , and 10 \times dose of PB (0.13, 1.3, and 13 mg/kg) alone or in combination with DEET (4, 40, and 400 mg/kg) and permethrin (0.013, 0.13, and 1.3 mg/kg). PB was administered, daily by gavage only on the last 15 days of the experiment. Our results show that treatment with PB alone led to significant impairment in the behavioral performance on all tests as compared to controls. There was no apparent dose effect on behavioral testing and no interaction between treatment group and dose level for the two behavioral tests for which this could be analyzed. The combination of PB and DEET as well as the combination of PB, permethrin, and DEET also led to poorer performance on each test. Combined exposure to PB and permethrin had a lesser impact. Furthermore, these data also suggest that treatment with PB, alone and in combination with DEET and permethrin, caused differential effects on AChE and m2 muscarinic receptors in the CNS.

The roles played by various anatomical brain regions and the molecular mechanisms involved in the behavioral effects observed in the present study are not known because these behavioral effects are mediated by a complex array of peripheral and central mechanisms. We have previously shown that exposure to DEET and permethrin at various doses alone and in combination causes neurobehavioral deficits (Abou-Donia et al., 2001a,b). Furthermore, our laboratory has also shown that 1 \times dose exposure to DEET and permethrin for 60 days causes neuropathological cell loss in somatosensory cortex and cerebellum (Abdel-Rahman et al., 2001). It is recognized that somatosensory and motor responses are mediated by three adjacent areas in the cortex. These are located in the granular, pyramidal, and posterior agranular layers of the cortex (Barth et al., 1990, Hurwitz et al., 1990, Kolb, 1984). Some of these areas respond to deep changes in tendons and muscles (Donoghue and Wise, 1982). Different lesion studies have shown that severe sensorimotor impairment occurs in the animals with lesions of anteromedial and caudal forelimb cortex (Barth et al., 1990). Similarly, studies with bilateral large lesions in the rat somatic sensorimotor cortex have shown impairment in limb placing response. Additionally, it has also been suggested that limb placing is a function of corticospinal tract (Hicks and D'Amato, 1975). Thus, it is possible that long-term treatment with various doses of PB alone or in combination with DEET or PB/DEET/permethrin could affect these innervations, leading to neurobehavioral deficits.

PB provides protection against organophosphate nerve agents by shielding the peripheral AChE by reversibly binding to it. Thus, the toxic effects of PB are thought to be mediated through peripheral nicotinic and muscarinic acetylcholine receptors (Albuquerque et al., 1997). Indeed, Chaney et al. (1999) found that PB induced seizures in the mouse were mediated via PNS muscarinic and nicotinic receptors. On the other hand, other studies also suggest that PB toxicity is mediated through CNS ACh receptors as well as through the PNS (Servatius et al., 1998). Consistent with the reported effects of PB in the PNS, our results indicate that PB treatment moderately inhibited plasma BChE activity, while the CNS AChE activity did not show any significant effects. Treatment with PB/DEET caused an increase in AChE activity that may be caused by an increase in AChE protein levels. PB, a cholinergic carbamate, may increase AChE expression, similar to that caused by sarin, a cholinergic organophosphate nerve agent (Damodaran et al., 2003). While not universally accepted, an increase in AChE protein may reflect an increased axonal repair and synaptic modeling (Bigbee et al., 2000; Sternfeld et al., 1998; Guizzetti et al., 1996). Therefore, it is possible DEET and permethrin treatment with PB/DEET may cause subtle changes that are reflected in increased synaptic modeling and repair. The behavioral observations following treatment with PB/DEET are consistent with this notion. The up-regulation of m2 muscarinic and nicotinic acetylcholine

receptor ligand binding by treatment with PB at each dose in our study is intriguing because PB does not cross the BBB. An alternate possibility is that there may be certain changes occurring at the cerebrovasculature endothelium leading to the passage of PB into the CNS. Although speculative, this amount of PB may not be sufficiently high to cause AChE inhibition, but it may cause the down-regulation of the muscarinic and nicotinic acetylcholine receptors, and as a compensatory mechanism, an increase in the ligand binding density. It is possible that PB entry into the CNS and the consequent inhibition of CNS AChE may enhance the toxic potential of neurotoxic agents. In the case of the CNS, however, combined exposure of PB with other potentially neurotoxic chemicals may prove additive based on its availability to inhibit AChE activity and a consequent regulation of muscarinic and nicotinic acetylcholine receptors. Furthermore, metabolic competition between these chemicals may also result in differential effects in the CNS, e.g., bioavailability of each of these compounds or their metabolites is affected by liver and plasma esterases that play a major role in metabolic inactivation of these compounds (Abou-Donia et al., 1996). This may explain why permethrin treatment negatively affected the effects of PB in our studies. Other studies also demonstrate that treatment with chemicals that cause inhibition of AChE lead to m2 muscarinic acetylcholine up-regulation (Witt-Enderby et al., 1995; Majocha and Baldessarini, 1984). The CNS effects of PB treatment alone are intriguing in that PB does not cross the BBB. Several recent studies suggested that PB could or could not elicit CNS effects in a variety of animals, such as mice (Friedman et al., 1996; Grauer et al., 2000), rat (Sinton et al., 2000; Li et al., 2000; Kant et al., 2001), and guinea pigs (Lallement et al., 1998; 2001). In our laboratory, we have observed that certain brain areas seem to be affected by PB more than the other areas. Since it is known that cholinergic system is present at the endothelial lining of the BBB vasculature, it is possible that local effects of PB on the BBB endothelial cells cholinergic pathway may enhance the delivery of PB in the CNS. However, this possibility needs further studies.

In summary, our results suggest that exposure to various doses of PB alone or in combination with DEET or a combination of PB, DEET, and permethrin resulted in sensorimotor deficits and alteration in the cholinergic system in rats. These changes may involve a combination of mechanisms related to central and peripheral or neuromuscular system. Such alterations may explain the symptoms and complaints of some of the veterans of the PGW.

Acknowledgements

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PHARMACOKINETIC INTERACTIONS BETWEEN DEET (*N,N*-DIETHYL-*m* -
TOLUAMIDE) AND PERMETHRIN FOLLOWING INTRAVENOUS AND DERMAL
DOSE IN RATS.

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Abstract

The pharmacokinetic interactions between a single dermal and intravenous (iv) dose of the insecticide permethrin (3-(2,2-dichloroethyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl) methyl ester) and the insect repellent DEET (*N,N*-diethyl-*m*-toluamide) has been investigated in rats. Rats were dosed dermal and intravenous with either 1.3 mg/kg (0.325 mg/1 cm² of skin area) of permethrin, and a dermal dose of 400 mg/kg (100 mg/ 1cm² of skin area) or an iv dose of 40 mg/kg of DEET, alone or in combination. Five rats dosed dermal were sacrificed at each time interval of 0.5, 1, 2, 4, 8, 24, 48, and 72 h after dosing. Plasma, liver, kidney, brain, testes, and urine collected and analyzed for permethrin, DEET, and their metabolites by high performance liquid chromatography (HPLC). In rats treated with a single agent, 0.5 and 72 h after dosing, the application site retained 45%, and 0.05% of DEET, and 62% and 4% of permethrin, respectively. Rate of distribution of permethrin in tissues was slower compared to DEET. At 8 hr after dosing, maximum concentrations of permethrin were 76 and 185 ng/g in liver and kidney, respectively. Permethrin metabolites *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid were identified in plasma, liver and kidney 24 h after administration. DEET and its metabolites *m*-toluamide and *m*-toluic acid were detected in plasma and tissues within 1 h of dosing. The time concentration curves of DEET and permethrin in plasma following dermal or iv dose were fitted to a one compartment pharmacokinetic model with a terminal half-life of elimination of 18.4, 32.6 h and 11.7, 22.9 h for DEET and permethrin, following iv and dermal dose, respectively. DEET and its metabolites *m*-toluamide and *m*-toluic acid were detected in urine samples before hydrolysis. Sequential enzymatic hydrolysis of urine samples showed that *m*-toluamyl

glucuronide and *m*-toluamyl sulfate conjugates were excreted in urine. Neither permethrin nor its metabolites *m*-phenoxybenzoic acid and *m*-phenoxybenzyl alcohol were detected in urine samples. Sequential enzymatic hydrolysis of urine samples yielded *m*-phenoxybenzyl alcohol, indicating that *m*-phenoxybenzyl glucuronide and *m*-phenoxybenzyl sulfate were excreted in urine following permethrin administration. Hot acid hydrolysis of urine samples yielded unidentified metabolites of DEET and permethrin.

Combined administration of both compounds either *iv* or dermal significantly increased AUC_{plasma} of DEET compared to AUC_{plasma} of DEET when applied alone. Permethrin significantly increased DEET half-life of elimination from plasma following combined *iv* administration. Concurrent *iv* administration of both compounds significantly increased permethrin AUC_{plasma} . But there was no significant effect on AUC_{plasma} of permethrin in the presence of DEET following dermal administration. There was no significant effect on absorption of either compound following dermal application of their combination compared to individual application. A significant decrease in the concentration of urinary conjugated metabolites of DEET following dermal combined application of both agents has been detected. The results showed that combined exposure to permethrin and DEET could prolong presence of DEET in the circulation system, reduce its rate of elimination and levels of conjugated metabolites.

Key words: DEET, permethrin, interactions, combined exposure.

Introduction

Permethrin is a pyrethroid insecticide effective against mites and head lice (Burgess et al., 1992; Fraser, 1994; Miller, 1989). DEET is a widely used insect repellent used against mosquitoes and other biting insects (USEPA, 1998; Robinson and Cherniak, 1986; Brown and Hebert, 1997). Both compounds were used during the Gulf War for pest control (Young and Evans, 1998). Permethrin modifies sodium channel to open longer during a depolarization pulse (Narahashi, 1985). Previous studies showed that DEET has direct effect on the nervous system in laboratory animals resulting in spongiform myelinopathy in the brain stem with signs including ataxia, seizures, coma, and death (Verschoyle et al, 1990). Furthermore, extensive and repeated topical application of DEET resulted in human poisoning including two deaths (Edwards and Johnson, 1987; Roland et al., 1985). Combined exposure to permethrin and DEET enhanced neurotoxicity in hens (Abou-Donia et al, 1996), modified the blood brain barrier in rats (Abou-Donia et al, 2001a, 2001b), caused oxidative stress in rats (Abu-Qare and Abou-Donia, 2000b, 2001b, 2001c; Abu-Qare et al, 2001), increased mortality in rats (McCain et al, 1998), and caused behavioral alterations in male rats (Abou-Donia et al., 2001a; Hoy et al, 2000a, 2000b). Published reports implicated exposure to permethrin and DEET in Gulf War illnesses (Abou-Donia et al., 1996; Haley and Kurt, 1997; Olsan et al., 1998; Kurt, 1998; Shen, 1998; Wilson et al., 1998; Jamal, 1998; Haley et al., 1999). Metabolism of permethrin has been examined following oral or *i.v* dose in rats (Abu-Qare and Abou-Donia, 2000a; Anadon et al, 1991). Disposition and metabolism of DEET has been studied *in vivo* following dermal application to human volunteers (Abu-Qare and Abou-Donia, 2000a; 2001c; Seliem et al., 1995; Blomquist

and Thorsell, 1977), in rats (Schoeing et al., 1996); in mice (Blomquist and Thorsell, 1977), in cattle (Taylor et al., 1994) and after topical application in beagle dogs (Qiu et al., 1997). DEET metabolism involved *N*-dealkylation, ring hydroxylation and ring dealkylation following *in vitro* incubation with rat liver microsomes (Constantino and Iley, 1999; Taylor, 1986).

We hypothesized that combined exposure to DEET and permethrin could be resulted in enhanced toxicity. A possible pathway of interactions between these compounds is affect on metabolism and pharmacokinetic of the other. This study reports on results of absorption, disposition, metabolism and excretion of single dermal doses of permethrin, or DEET, alone and in combination in rats.

Materials and methods

Chemicals

Permethrin (99%, (3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl)methyl ester) was purchased from Chem Service Inc. (West Chester, PA). *m*-Phenoxybenzyl alcohol, *m*-phenoxybenzoic acid, β -glucuronidase from bovine liver type B-1, and sulfatase from *limpets* type V were obtained from Sigma Chemical Co., (St. Louis, MO). DEET ($\geq 97\%$ *N,N*-diethyl-*m*-toluamide) was obtained from Aldrich Chemical Co, Inc. (Milwaukee, WI). *m*-Toluamide, and *m*-toluic acid were purchased from Fisher Scientific (Pittsburgh, PA). C_{18} Sep-Pak^R cartridges were obtained from Waters Corporation (Waters Corporation, Milford, MA). Water and acetonitrile (HPLC grade) were obtained from Mallinckrodt Baker, Inc. (Paris, KY).

Chromatographic conditions

The liquid chromatographic system (Waters 2690 Separation Module), consisted of a Waters 600E multisolvent delivery system pumps, a Waters Ultra WISP 715 autoinjector and a Waters 2487 dual λ absorbance detector (Waters Corporation, Milford, MA). A guard column (Supelco, 2 cm \times 4.0 mm, 5 μ m (Supelco Park, Bellefonte, PA), and a reversed-phase C_{18} column μ Bondapak C_{18} 10 μ m, 3.9 \times 300 mm were used, (Waters Corporation, Milford, MA) were used. The mobile phase was water (adjusted to pH 3.0 using 0.1N acetic acid): acetonitrile gradient at flow rate ranging between 1.0-1.7 ml/min. The gradient started at 1% acetonitrile, increased to 25% acetonitrile at 3.6 min, then to 45% acetonitrile at 6 min, and up to 85% acetonitrile at 11 min. The system returned to 1% acetonitrile at 14 min and was kept under these conditions for 3 min to re-equilibrate.

Total run time was 17 min. The analytes were monitored by UV detection at 210 nm for permethrin, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, and 230 nm for DEET, *m*-toluamide, and *m*-toluic acid. The chromatographic analysis was performed at ambient temperature (Figure 1).

Calibration procedures, detection limits and recoveries

Five different calibration standards of a mixture of permethrin, DEET, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid were prepared in acetonitrile. Their concentrations ranged from 100-1000 ng/ml following a method developed and validated in our laboratory (Abu-Qare and Abou-Donia, 2000a). Linear calibration curves were obtained by plotting the peak areas of the individual chemicals as a function of concentration. The standard curves were used to determine recovery of the chemicals from plasma and tissue samples. Limits of detection were determined at the lowest concentration that can be detected, taking into consideration a 1:3 baseline noise: calibration point ratio. Plasma, urine and tissue samples from control animals were spiked with selected concentration ranging between 100-1000 ng/ml of each permethrin, DEET and metabolites (Abu-Qare and Abou-Donia, 2000a). The samples were extracted and cleaned up as described under sample preparation. Percentage recoveries of each compound were determined using the calibration curves as described above.

Animals treatment and handling

Sprague Dawley rats (250-300 g) were purchased from Zivic Miller (Zelienople, PA). The animals were kept in a 12-h light/dark cycle (temperature 21-23°C) and were provided with a free supply of feed (Rodent Laboratory Chow, Ralston Purine Co., St. Louis, MO) as well as tap water. Animal care was conducted according to institutional guidelines. Permethrin and DEET were dissolved in 70% ethanol. A single dose of 1.3 mg/kg of permethrin or 400 mg/kg of DEET, or both doses in combination (DEET followed by permethrin) were applied with a micropipette (1 ml/kg) to an unprotected 1 cm² area of pre-clipped skin on the back of each rat. The application area was not protected to resemble real-life situations. A group of five animals was used for each time point. The doses were selected to represent real-life exposure levels (Abou-Donia et al., 1996; 2001a, 2001b). Five control animals were treated with equal volume of 70% ethanol either to resemble single application (250 µl) or combined application (500 µl) and kept under similar conditions as treated rats. Rats were placed into metabolic cages after dosing to facilitate urine collection and five rats per time point were anesthetized with halothane (Halothane Laboratories, River Edge, PA) at intervals of 0.5, 1, 2, 4, 8, 24, 48, and 72 hr after dosing and exsanguinated by cardiac puncture into heparinized syringes. Samples of liver, kidney, brain and testes were collected from each animal and portion of the blood volumes were separated into plasma samples by centrifugation at 2400 rpm for 10 min at 5°C. Plasma and tissue samples were stored at -70°C until analysis

Sample preparation

Plasma (0.5 ml), and urine (1.0 ml) sample, or 0.5 g of liver, kidney, brain, and testes were each mixed or homogenized with a 2 ml of (1:1) of acetonitrile and methanol, centrifuged at 1000g for 5 min, and the supernatant was removed. Disposable C₁₈.

Sep-Pak Vac 3cc (500mg) cartridges were conditioned with 3 ml of acetonitrile, then equilibrated using 3 ml of water prior to use. The supernatant was loaded into the disposable cartridges, washed by 3.0 ml of water, followed by elution with 2 ml of methanol, and 2 ml of acetonitrile. The elution volume was reduced to 500 μ l (0.5 ml) in a test tube rack using a gentle stream of nitrogen, prior to analysis by HPLC.

Urine Analysis

Non-conjugates

A portion of each urine sample (1 ml) was acidified using 0.1N phosphoric acid (pH 4) and passed through Sep pack cartridges as described above. The contents were analyzed using HPLC to determine parent compound and non-conjugated metabolites. Another portion of the same urine sample (1ml) was used for determination of the conjugates as describe below.

Conjugates

(a) Enzymatic hydrolysis

i) Glucuronides. β -glucuronidase from bovine liver type B-1 was added to urine residues in 0.2 M sodium acetate buffer (pH 4.5), incubated for 18 hours at 37°C, then the urine incubate was analyzed as previously described under sample preparation,

(ii) Sulfates. The remaining urine residue was incubated for 24 hours at 37°C with sulfatase from *limpets* type V in 0.2 M sodium acetate buffer (pH 4.5). The residue was analyzed as described above under sample preparation.

(b) Hot acid hydrolysis

Another 1.0 ml urine sample was adjusted by 1 N sulfuric acid to (pH 2.5), heated for 2 hours at 80°C, and analyzed as described above.

Kinetics analysis

The kinetic analysis of permethrin, DEET and metabolites in plasma and tissues were performed using WinNonlin Program (Pharsight Corporation, Mountain View, CA). The terminal half-life of permethrin, DEET and their metabolites was calculated from the elimination rate constant K_{10} , that was obtained by linear regression of the terminal linear exponential decline in concentration. The total area under the compound concentration vs. time curves for plasma, AUC_{plasma} , or brain AUC_{brain} was calculated by the trapezoidal rule and extrapolated to infinity by using the last data point and the respective terminal linear experimental decline.

Statistical analysis

The pharmacokinetic parameters were subjected to analysis of variance (ANOVA) to determine if the difference between treatment with the individual compound and the combination is significance using a GraphPad Prism program for Windows (GraphPad

Software, Inc., San Diego, CA). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Clinical observation

A single dermal dose of 1.3 mg/kg of permethrin, or a single dermal dose of 400 mg/kg of DEET, or both agents at these doses in combination did not produce observable toxic effect in rats, based on gross examination without morphological or physiological studies carried out. Both control and treated animals consumed comparable amounts of feed and water. There was no difference in weight, size, shape, or color of various tissues of treated animals compared to tissues of control rats.

Limits of detection and recovery of permethrin, DEET, and metabolites

Average percentage recoveries of permethrin, DEET, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid from plasma were 82.3 ± 8.2 , 71.1 ± 11.2 , 73.6 ± 10.1 , 79.3 ± 8.5 , 84.3 ± 12.0 , and 82.6 ± 6.1 , respectively. Their limits of detection were 30, 50, 50, 80, 20, and 30 ng/ml, respectively.

Disappearance of permethrin and DEET from the application site

Permethrin and DEET were disappeared at different rate from rat skin following a single dermal dose. The application site retained 62% and 4% of permethrin, and 45% and 0.05% of DEET after 0.5 hr and 72 hr following application, respectively. Rate of disappearance of both compounds was more rapid during the early time after application, then gradually decreased (Table 1)

DEET and permethrin in plasma and tissues

Tables 2 and 3 list the concentrations of permethrin and DEET (ng /g fresh tissue or mg/ ml plasma) at different time intervals following a single dermal dose of both chemicals in rats. Permethrin, DEET and their metabolites were distributed and detected in analyzed tissues. Permethrin was found in the kidney, liver, brain and plasma. Maximum concentration of DEET was also detected in the analyzed tissues in the following order kidney > liver > plasma > brain > testes. Following administration of a combined dose of permethrin and DEET, higher amount of DEET was detected in plasma (Table 4), while there was no significant change in the pattern of distribution and amount of permethrin and its metabolites when it was concurrently applied with DEET (Table 5).

Metabolism of permethrin and DEET

Metabolites of permethrin and DEET were analyzed using high performance liquid chromatography (HPLC) (Fig 1). *m*-Phenoxybenzyl alcohol, a metabolite of permethrin, was detected in liver, while *m*-phenoxybenzoic acid was detected in liver, kidney and plasma over the time course of analysis. DEET metabolites *m*-toluic acid and *m*-toluamide were found in all analyzed tissues and plasma (Tables 2 and 3). Proposed metabolic pathways of permethrin and DEET are shown in Figures 2 and 3.

Urinary excretion of DEET and permethrin following dermal dose

Urinary excretion was rapid following dermal application of DEET. DEET and its metabolites *m*-toluamide and *m*-toluic acid were identified in urine shortly after application. Following enzymatic hydrolysis with glucuronidase, level of DEET

metabolites increased, indicating that *m*-toluamyl and toluyyl glucuronide conjugates were excreted in the urine (Table 6). Incubations of urine samples with sulfatase resulted in a significant increase in the concentrations of both metabolites, a further indication of existence of *m*-toluamyl sulfate and *m*-tolyl sulfate conjugates (Table 6). Urinary excretion of permethrin metabolites was slower compared to DEET. A similar trend was detected following incubation of permethrin urinary excretion with glucuronidase and sulfatase. *m*-Phenoxybenzyl alcohol was detected (Table 7). Acid hydrolysis of urine samples produced unidentified metabolites for both DEET and permethrin.

Bioavailability of DEET and permethrin.

Plasma concentrations of DEET, permethrin and their metabolites following *iv* dose are shown in tables 8 and 9. The systemic bioavailability of DEET was 45% and 40% when administered alone and in combination with permethrin, respectively (Table 10). Permethrin bioavailability was 22% and 16% when applied alone or in combination with DEET, respectively (Table 11).

Pharmacokinetics profiles of permethrin, DEET.

Permethrin and DEET disappeared mono and bi-exponentially from plasma following a single dermal and *iv* dose in rats, respectively. The time concentration curves of permethrin and DEET in plasma were fitted to one and two compartment model, respectively. The pharmacokinetic parameters of permethrin, DEET are listed in Table 10 and 11.

Pharmacokinetic interactions

Concurrent administration of permethrin and DEET had no significant effect on rate of disappearance of either compound from the site of application (Table 1). Pharmacokinetic profiles of permethrin, and DEET following *iv* or dermal combined administration revealed that permethrin significantly increased DEET AUC_{plasma} (Figures 4 and 6). Also an *iv* dose of permethrin significantly increased DEET half-life of elimination from the plasma (Table 10). DEET significantly increased the AUC_{plasma} of permethrin following *iv* concurrent administration (Figure 5). But there was no significant effect on permethrin pharmacokinetic profiles following dermal concurrent application with DEET (Figure 7). The concurrent application of both compounds decreased rate and levels of DEET urinary excretion (Figure 8).

Discussion

A single dermal dose of 400 mg/kg of DEET was rapidly disappeared from the application site and distributed in tissues after administration in rats. These results are in agreement with previous reports on absorption and disposition of DEET in rats (Schoenig et al., 1996), in mice (Blomquist and Thorsell, 1977), in beagle dogs (Qiu et al., 1997), and following dermal application to human volunteers (Seliem et al., 1995). Furthermore, DEET has been shown to rapidly cross the dermal barrier following topical application in rats (Windhenser et al., 1982). In the present study DEET was applied in ethanol. Its pattern of absorption could be different if applied in other carrier vehicles. Qiu et al. (1998) reported that *in vitro* skin permeation, the vehicle showed marked effect on rat skin penetration of DEET. Absorption of DEET was significantly correlated to the type of commercial formulation following application in rats (Stinecipher and Shah, 1997). Our findings showed that while 11% of the applied dose of DEET was retained by the application site 24 h after application, more than 99% of the applied dose of DEET disappeared from the application site 72 h after application. These data are consistent with those reported by Schoenig (1996), that 16% of the dose was retained by the application site 36 hr after dermal dose of DEET in human volunteers and by Taylor et al. (1994) that 72.9% of DEET was absorbed into the systemic circulation following dermal single dose in cattle.

DEET was distributed rapidly in the tissues analyzed with a maximum concentration found within 4-8 hr after application; liver>kidney>plasma>brain>testes. In previous study, Blomquist and Thorsell (1977) reported that blood, kidney and liver

had high concentration of radioactivity after cutaneous application of ^{14}C -*N,N*-diethyl-*m*-toluamide in mice.

Disappearance of permethrin from the application site was slower compared to DEET, possibly due to differences between the compounds in their molecular weights and physical properties such as lipid solubility. Only 4% of administered dose of permethrin was retained by the application site after 72 h. In addition to its absorption by rat skin, permethrin and DEET could be dissipated from rat skin through other pathways such as volatilization, or through contact with surfaces of metabolic cages.

Permethrin and metabolites were detected in tissues and plasma within 0.5-24 hr after application. The findings are consistent with the results of Anadon et al (1991) who reported that permethrin was distributed in tissues and accumulated in the nervous system following an oral dose of 460 mg/kg in rats.

Figures 2 and 3 present a suggested scheme for the metabolism of dermal dose of DEET and permethrin. DEET metabolites *m*-toluamide and *m*-toluic acid were detected in some of the analyzed tissues. In this study, DEET underwent metabolic transformation mediated by esterases and cytochrome p450 enzymes, resulted in formation of metabolites of *m*-toluic acid and *m*-toluamide. This is in agreement with previous reports following dermal dose of DEET in rats and dogs (Schoenig et al, 1996; Qiu et al, 1997), and in accordance with previous studies reported that DEET metabolism mediated by *N*-dealkylation, ring hydroxylation and ring dealkylation following *in vitro* incubation with rat liver microsomes (Constantino and Iley, 1999; Taylor, 1986). These findings are also

consistent with our recent results obtained following *in vitro* incubation of DEET with human liver microsomes (Abu-Qare and Abou-Donia, 2001d).

In this study, DEET metabolite *m*-toluic acid was detected in plasma 1 hr after application indicates the rapid hydrolysis of DEET *in vivo*. Permethrin and its metabolites *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid were also detected at 2 h after application in plasma and tissues, showing the role of esterases in its metabolism. In previous results, permethrin metabolites *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid were detected in plasma and tissues up to 48 h after an oral dose in rats (Anadon et al, 1991). Metabolism of permethrin reported in this study indicates its hydrolytic cleavage as an initial phase of metabolism. Rapid hydrolysis of permethrin was catalyzed by butyrylcholinesterases following *in vitro* incubation with human plasma, where addition of butyrylcholinesterase inhibitor Iso-OMPA significantly inhibited permethrin metabolism *in vitro* (Abu-Qare and Abou-Donia, 2001d). Furthermore, permethrin metabolism proceeded through oxidation and the formation of *m*-phenoxybenzoic acid. This is in accordance with a report that permethrin oxidative pathway is mediated by cytochrome P450 enzymes, thus more toxicity has been detected when an oxidase inhibitor PB (piperonyl butoxide) was applied with permethrin in rats (Vulule et al, 1999). In other study, permethrin induced cytochrome P-4502B in rat liver (Koska et al, 1997).

The present results suggest that the pharmacokinetics analysis of DEET following a single dermal dose of 400 mg/kg would require a one-compartment open-model system. Elimination half-life of DEET was 32 h, while T_{max} in plasma was 2.1 h and C_{max} was

2103 ng/ml. In their study, Hoy et al. (2000) reported that blood serum concentration of DEET was 12347 ng/ml following an i.p injection of 500 mg/kg in male rats. In another study, DEET's elimination half-life was 200.2 min following a dermal dose in human volunteers (Schoeing, 1996). Following its administration, only small amount of the intact DEET was excreted in the urine. Majority of the urinary excretion following a single dermal dose of DEET and permethrin was in the form of conjugated metabolites. This was confirmed following sequential enzymatic hydrolysis with glucuronidase and sulfatase.

The results also suggest that permethrin is also fitted to one compartment pharmacokinetic model, with half-life of elimination from plasma of 22.9 h. This is in accordance with Anadon et al (1991) who suggested one compartment model for the plasma profile of permethrin following oral dose of 460 mg/kg or *i.v* dose of 46 mg/kg in rats, where elimination half-life of permethrin was from rat plasma following an oral dose was 12.37 hr.

The results show that dermal administration of a combined dose of DEET and permethrin resulted in a significant increase in the AUC_{plasma} of DEET in rats, with a significant increase in its half-life of elimination following *iv* dose, and a decreased rate of its urinary excretion. In contrast to the effect of permethrin on DEET in rat plasma, DEET did not significantly affect permethrin kinetics after dermal administration. This could be due to the low dose of permethrin used compared to DEET, and to a rapid hydrolytic cleavage of permethrin, presumably by esterases. This trend of rapid cleavage of permethrin has been reported *in vivo* and *in vitro* incubation with human plasma (Abu-Qare and Abou-Donia, 2000a; Abu-Qare and Abou-Donia, 2001d). Moss (1996) also

reported that DEET inhibited hydrolysis of some but not all of cholinesterase inhibitors in German cockroaches.

In this study, DEET and permethrin were applied in 70% ethanol, and at different application sites on the back of the neck of rats. This was to avoid possible interaction at the application site. The findings suggest there was no significant effect of each compound on absorption of the other. Our findings of *in vivo* disappearance of Coadministered DEET and permethrin do not agree with previous *in vitro* studies in which DEET was administered concomitantly with another insecticide or drug. When both DEET and permethrin were coadministered to rodent and pig skin *in vitro*, DEET significantly decreased permethrin absorption (Baynes et al., 1997), and significantly enhanced persistence of the pyrethroid insecticide fenitrothion by rat skin *in vitro* (Moody et al, 1987). Furthermore, addition of DEET to the gel of the drug methotrexate resulted in two-fold increased permeation of methotrexate into muscle within 4 h after dosing in rabbits (Lu et al., 1997). The gradual decrease in rate of disappearance of DEET and permethrin starting 24 h after application could be due to the saturation of the application site at that time, or to their binding to skin constituents.

In summary, co-administration of permethrin and DEET increased DEET concentration in the plasma, decreased its urinary excretion, and enhanced its persistence in the body as evident from AUC_{plasma} following combined application. This effect may be due to a competition between permethrin and DEET on certain metabolic pathway, thus reducing the DEET rate of metabolism. In a recent study in our laboratory, permethrin inhibited DEET dissipation following *in vitro* incubation by human liver microsomes (Abu-Qare and Abou-Donia, 2001d). In a previous study by Hoy et al. (2000a),

cutaneous administration of 15 mg/kg of permethrin with 5 mg/kg of pyridostigmine bromide increased permethrin concentration in serum of male and female rats compared to permethrin when applied alone at rate of 30 mg/kg.

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Figure legends

Figure 1 HPLC profile of DEET, permethrin and their metabolites A) *m*-toulamide, B) *m*-toluic acid, C) DEET, D) *m*-phenoxybenzyl alcohol, E) *m*-phenoxybenzoic acid, and F) permethrin.

Figure 2 Suggested metabolic pathways of DEET following a single dermal dose in rats.

Figure 3 Proposed metabolic pathways of permethrin following a single dermal dose in rats.

Figure 4 Time course changes of DEET concentration in plasma of rats after a single intravenous dose, alone and in combination with permethrin.

Figure 5 Time course changes of permethrin concentration in plasma of rats after a single intravenous dose, alone and in combination with DEET .

Figure 6 Time course changes of DEET concentration in plasma of rats after a single dermal dose, alone and in combination with permethrin. .

Figure 7 Time course changes of permethrin concentration in plasma of rats after a single intravenous dose, alone and in combination with DEET.

Figure 8 Urinary excretion of DEET and metabolites after a dermal single dose, alone and in combination with permethrin.

Figure 9 Urinary excretion of permethrin metabolites after a dermal single dose, alone and in combination with DEET.

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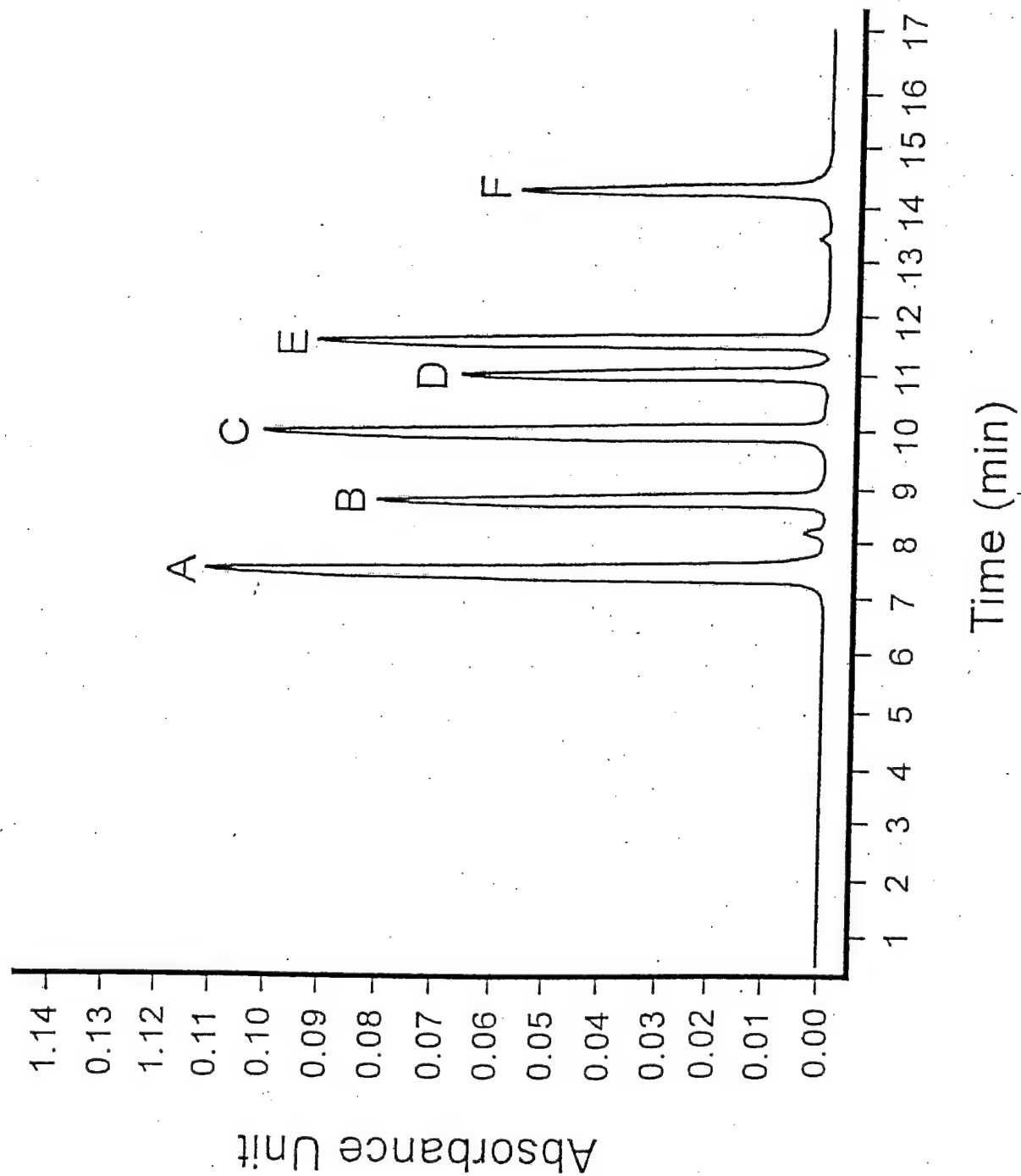


Fig. 1

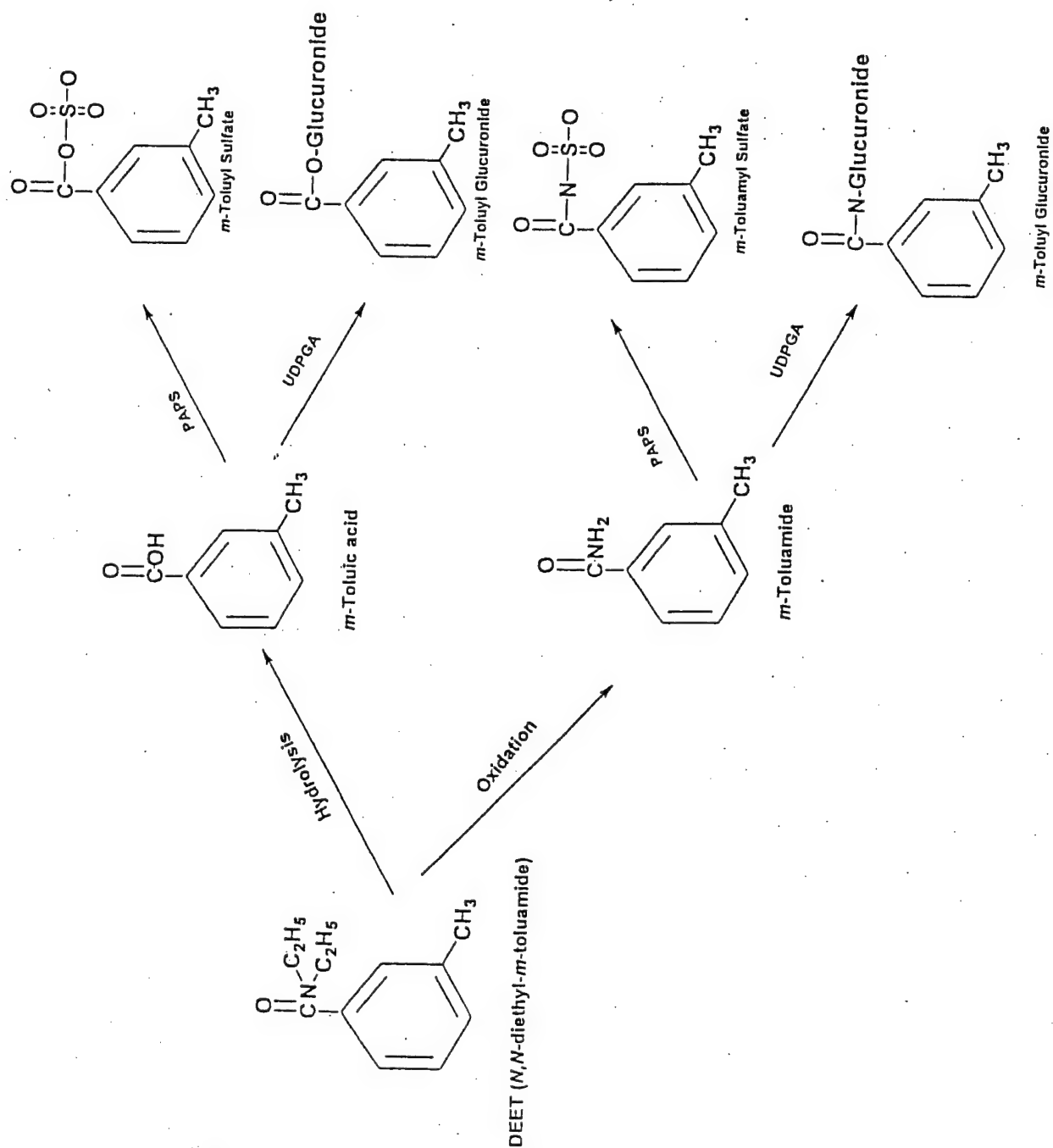


Fig. 8

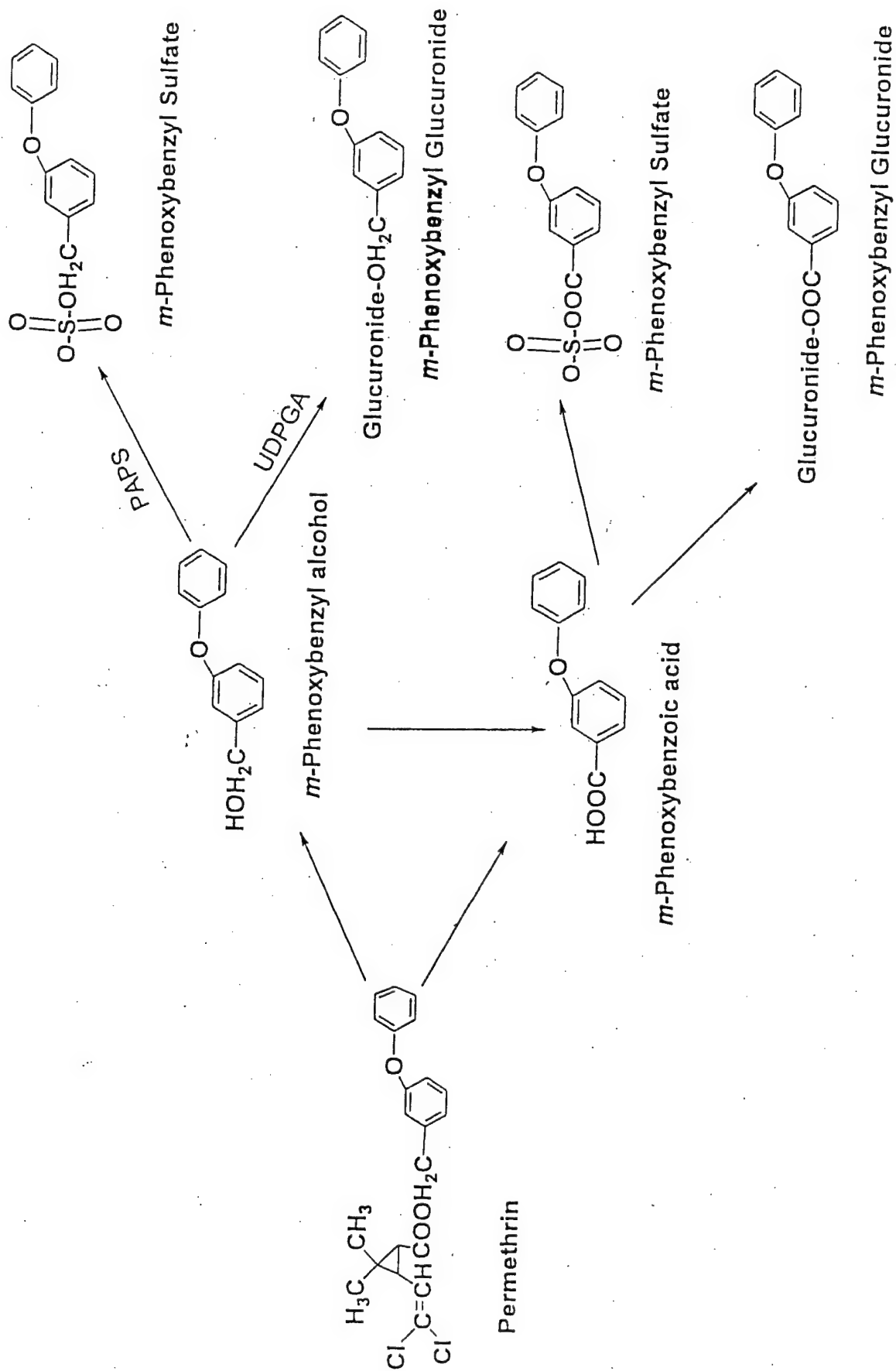


Fig. 1

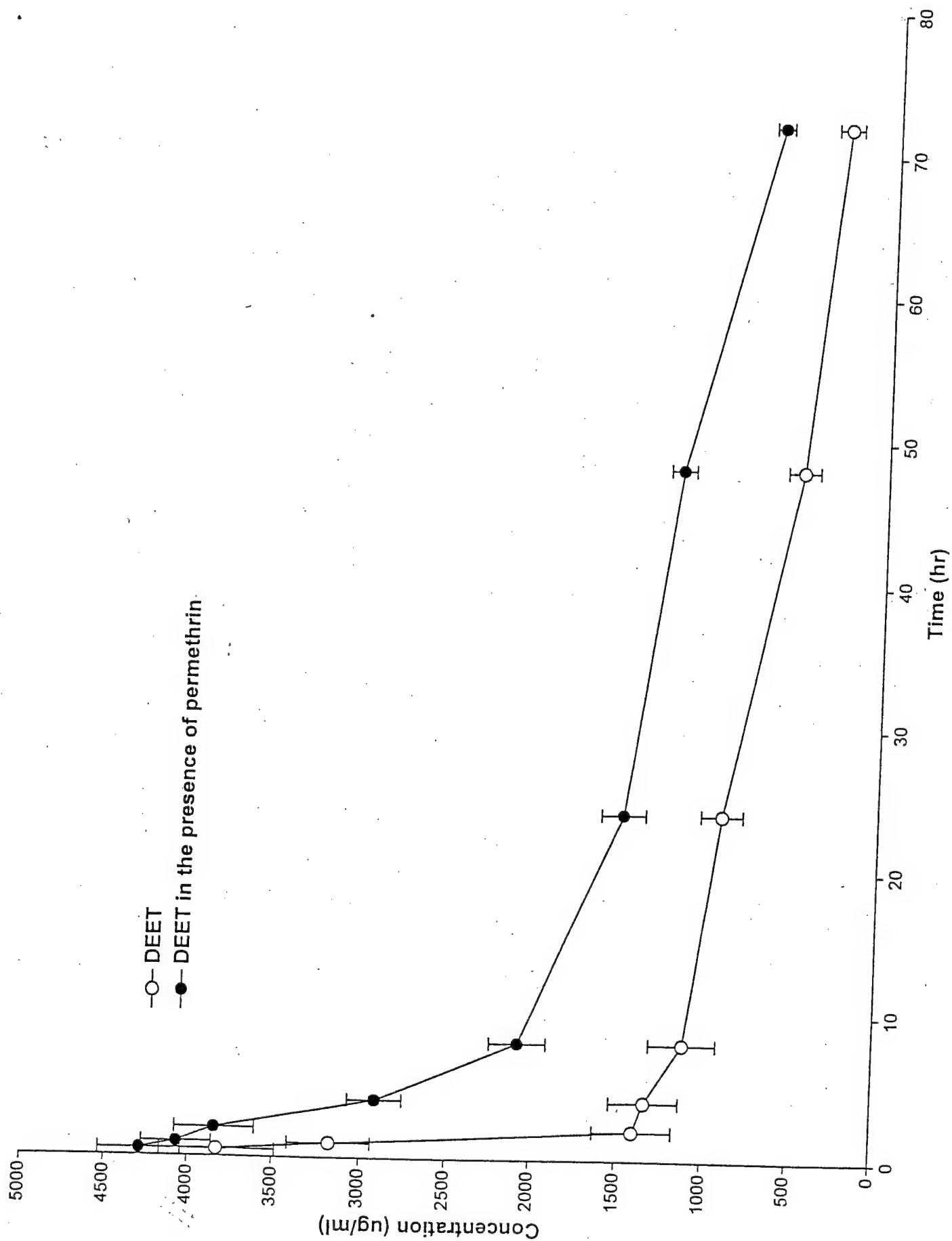


Fig. 5

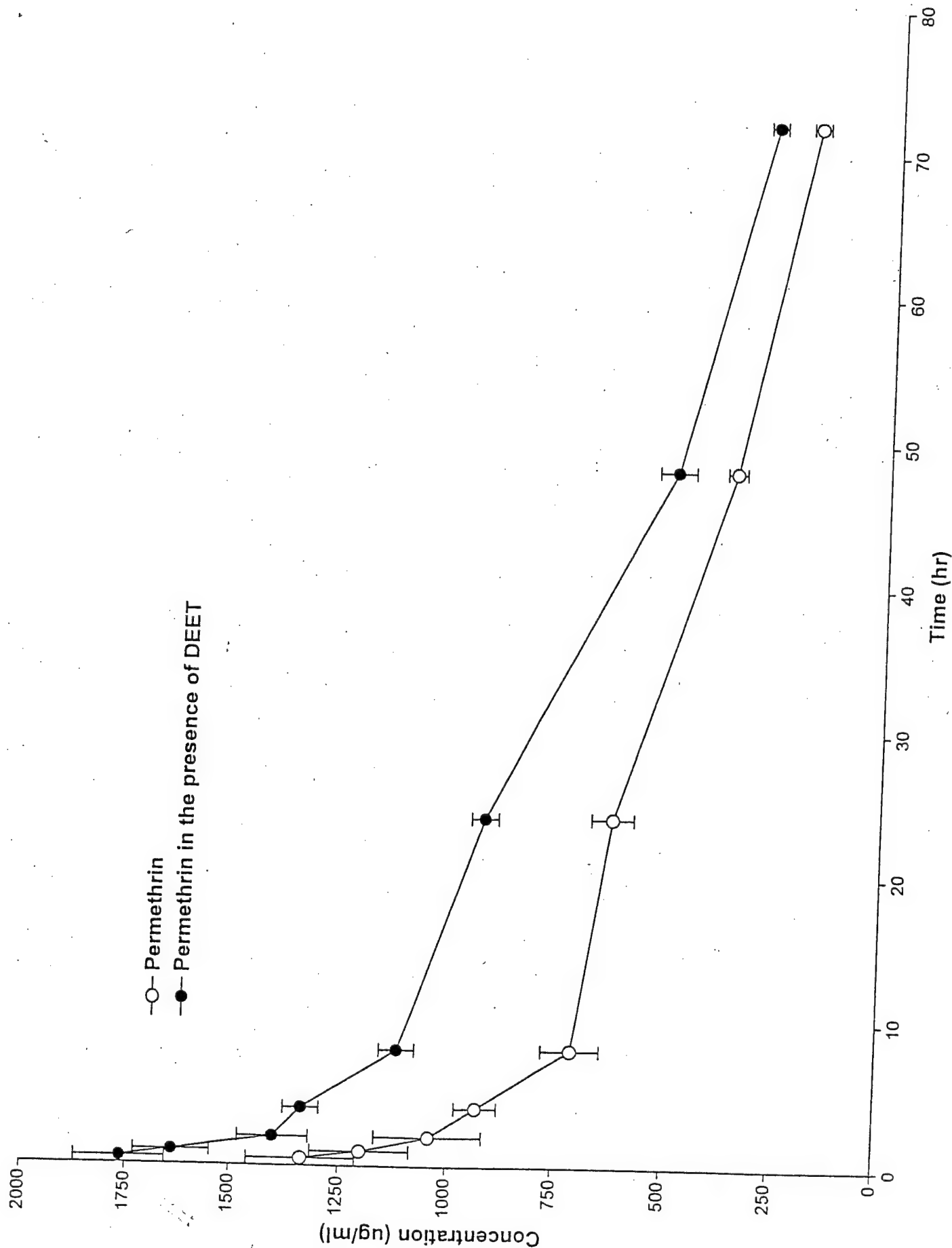


Fig. 6.

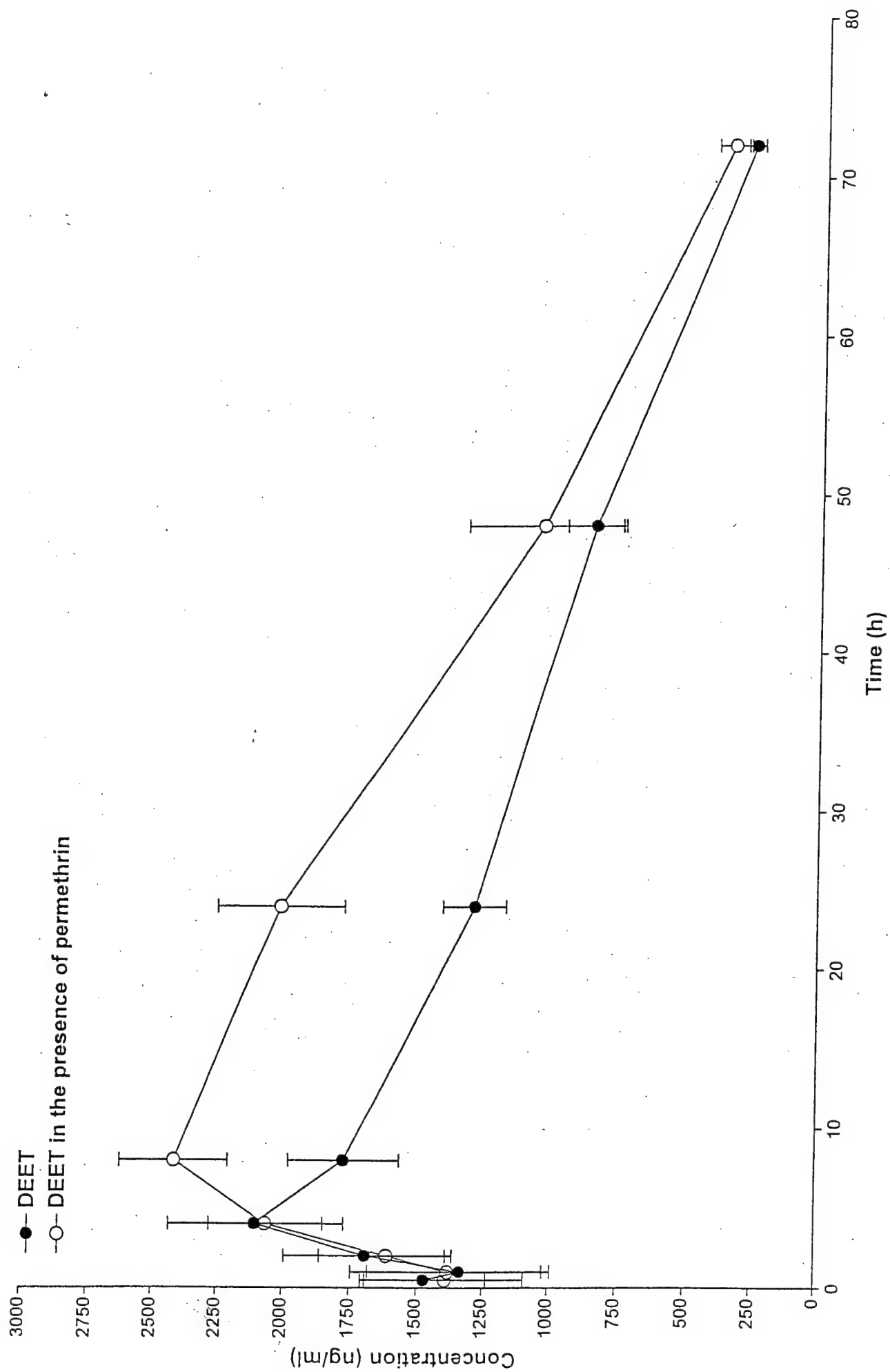
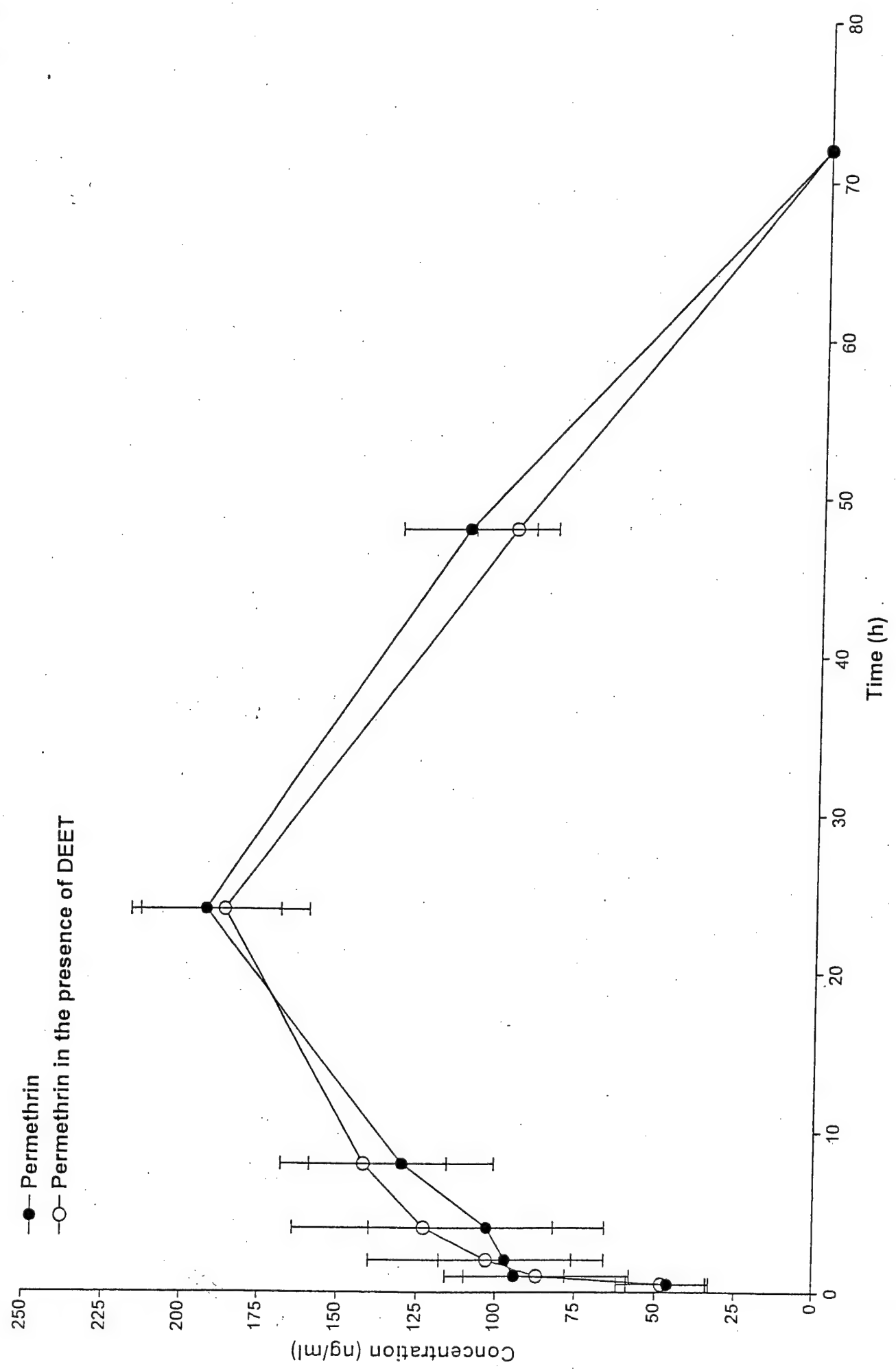


Fig. 7



The bar chart displays the concentration (ng/ml) of ten different compounds over a period of 72 hours. The y-axis represents concentration from 0 to 450 ng/ml, and the x-axis represents time in hours from 0.50 to 72.00. The compounds are listed in the legend: DEET, DEET with permethrin, *m*-Toluamide, *m*-Toluamide-permethrin, *m*-Toluic acid, *m*-Toluic acid-permethrin, Glucuronide conjugates, Glucuronide conjugates-permethrin, Sulfate conjugates, and Sulfate conjugates-permethrin. Error bars are present for each data point. Asterisks (*) above certain bars indicate statistically significant differences compared to the control or other groups.

Compound	0.50 hr	1.00 hr	2.00 hr	4.00 hr	8.00 hr	24.00 hr	48.00 hr	72.00 hr
DEET	~50	~40	~40	~100	~100	~260	~260	~410*
DEET with permethrin	~40	~40	~40	~120	~120*	~210	~210	~230*
<i>m</i> -Toluamide	~40	~40	~40	~100	~100	~260	~260	~410*
<i>m</i> -Toluamide-permethrin	~40	~40	~40	~100	~100	~260	~260	~410*
<i>m</i> -Toluic acid	~40	~40	~40	~100	~100	~260	~260	~410*
<i>m</i> -Toluic acid-permethrin	~40	~40	~40	~100	~100	~260	~260	~410*
Glucuronide conjugates	~40	~40	~40	~100	~100	~260	~260	~410*
Glucuronide conjugates-permethrin	~40	~40	~40	~100	~100	~260	~260	~410*
Sulfate conjugates	~40	~40	~40	~100	~100	~260	~260	~410*
Sulfate conjugates-permethrin	~40	~40	~40	~100	~100	~260	~260	~410*

Fig. 9

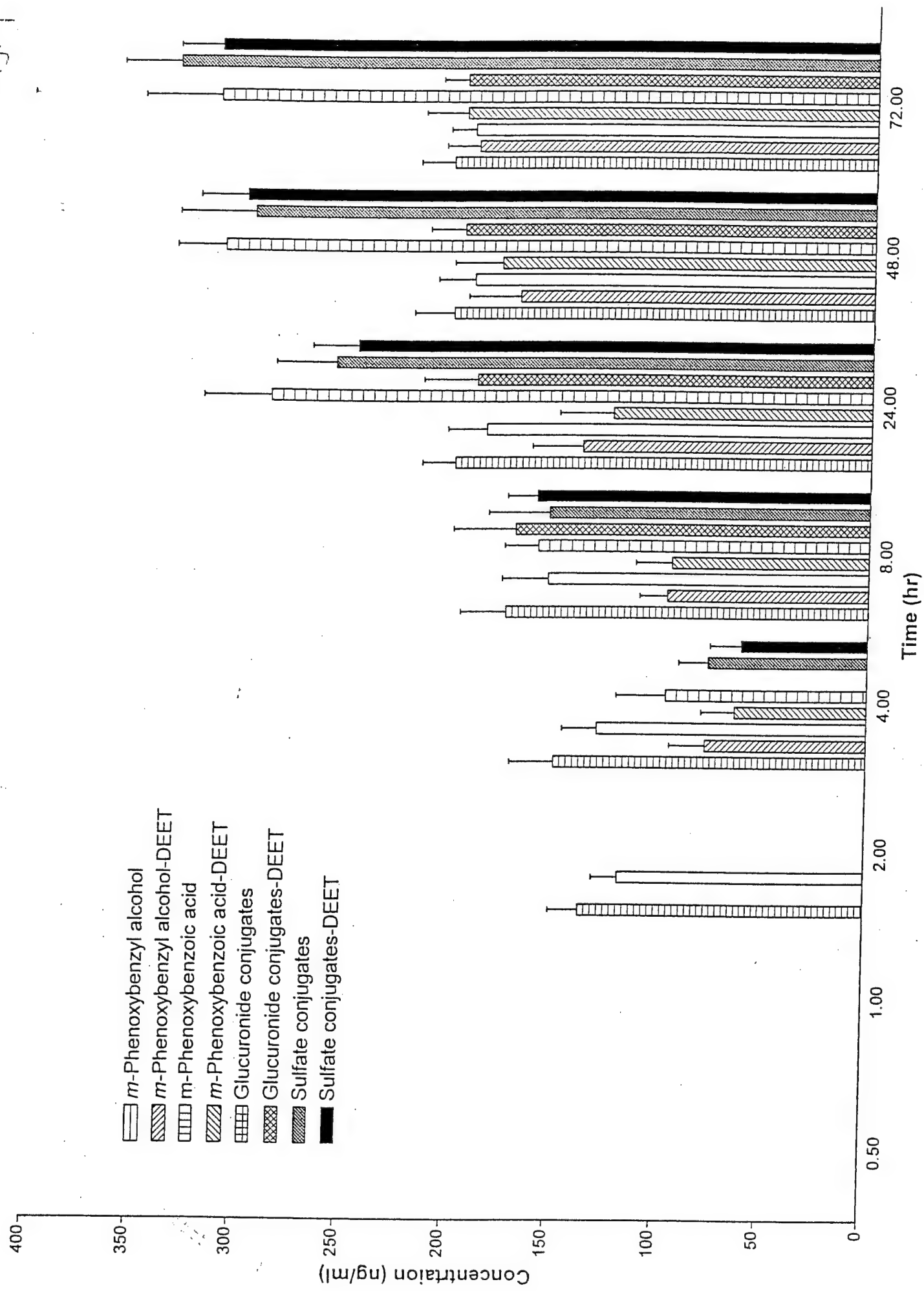


Table.1 Retention^a of DEET^b and permethrin by rat skin following a single dermal dose of 400 mg/kg of DEET, and 1.3 mg/kg of permethrin, alone and in combination in Sprague-Dawley rats.

Time (h)	DEET	Permethrin	DEET in the presence of permethrin	Permethrin in the presence of DEET
0.5	45.6±7.8	62.3±13.4	51.3±9.0	72.6±8.5
1	32.7±12.6	57.8±10.9	43.8±9.8	65.6±6.7
2	28.6±6.5	49.2±7.6	36.5±5.6	54.6±7.2
4	22.1±8.2	38.9±12.6	30.1±8.7	45.6±7.2
8	18.7±9.6	29.7±6.3	25.6±9.0	33.7±5.9
24	11.2±5.6	21.1±10.9	18.6±5.6	18.9±7.0
48	1.5±1.2	11.7±5.4	5.3±4.5	9.1±4.6
72	0.05±0.03	4.2±1.5	0.5±0.3	6.1±3.0

^a Results are expressed as percentage of the applied dose (mean±SD) of five animals.

^b DEET and permethrin were applied in ethanol.

Table.2 Tissue concentration^a of DEET and metabolites following a single dermal dose of 400 mg/kg in Sprague-Dawley rats.

DEET							<i>m</i> -Toluic acid			<i>m</i> -Toluamide		
Time (h)	Plasma	Liver	Kidney	Brain	Testes		Plasma	Liver	Kidney	Plasma	Liver	Kidney
0.5	1472±361	921±219	891±721	241±101	N.D		241±65	202±131	98±73	132±96	169±102	34±26
1	1536±645	1204±532	956±207	247±123	N.D		313±148	119±75	112±76	245±93	301±163	101±64
2	1792±823	1650±302	1460±372	351±112	N.D		401±230	102±56	134±82	387±128	486±321	183±58
4	2103±631	1832±534	1609±237	260±134	N.D		391±90	187±69	174±101	460±153	512±201	290±259
8	1776±405	2209±709	1967±452	210±108	N.D		301±113	112±64	110±32	512±234	601±187	124±23
24	1294±320	1645±497	1830±643	198±93	181±54		109±76	N.D	125±68	460±254	245±123	N.D
48	852±209	1034±420	1273±297	76±34	165±63		N.D	N.D	N.D	N.D	N.D	N.D
72	276±132	354±98	675±245	N.D	N.D		N.D	N.D	N.D	N.D	N.D	N.D

^a Results are expressed as ng/g fresh tissue or ml plasma (mean± SD) of 10 samples from five animals (two samples/animal).
^b DEET was dissolved in ethanol.

Table.3 Tissue concentration^a of permethrin^b and metabolites following a single dermal dose of 1.3 mg/kg in Sprague-Dawley rats.

Permethrin							<i>m</i> -Phenoxybenzyl alcohol				<i>m</i> -Phenoxybenzoic acid		
Time (h)	Plasma	Liver	Kidney	Brain	Testes		Plasma	Liver	Kidney		Plasma	Liver	Kidney
0.5	46±13	N.D	N.D	N.D	N.D		N.D	N.D	N.D		N.D	N.D	N.D
1	94±16	N.D	148±42	N.D	N.D		N.D	N.D	N.D		N.D	N.D	N.D
2	97±41	65±27	153±29	N.D	N.D		56±12	N.D	42±19		102±13	73±8.6	N.D
4	103±37	59±31	183±101	N.D	N.D		93±31	71±23	56±13		97±9.5	82±17	N.D
8	130±29	76±35	185±92	49±32	N.D		57±10	68±41	59±20		83±16	89±26	72±9.2
24	193±54	63±12	109±63	52±27	N.D		N.D	63±19	73±16		132±52	126±40	63±13
48	112±51	N.D	73±31	N.D	N.D		N.D	78±36	68±23		121±26	123±28	78±21
72	N.D	N.D	68±29	N.D	N.D		N.D	N.D	49±21		N.D	68±13	N.D

^a Results are expressed as ng/g fresh tissue or ml plasma (mean± SD) of 10 samples from five animals (two samples/animal).

^b Permethrin was dissolved in ethanol.

Table.4 Tissue concentration^a of DEET^b and metabolites following concurrent application of a single^c dermal dose of 400 mg/kg of DEET, and 1.3 mg/kg of permethrin in Sprague-Dawley rats.

DEET										<i>m</i> -Toluic acid				<i>m</i> -Toluamide			
Time (h)	Plasma	Liver	Kidney	Brain	Testes	Plasma	Liver	Kidney	Kidney	Plasma	Liver	Kidney	Kidney	Plasma	Liver	Kidney	Kidney
0.5	1391±601	1126±349	931±218	301±97	N.D	183±76	213±138	113±29	113±29	189±18	156±53	91±63	91±63	189±18	156±53	91±63	91±63
1	1382±362	1163±521	1073±308	273±101	N.D	291±118	224±109	146±53	146±53	269±87	297±101	176±23	176±23	269±87	297±101	176±23	176±23
2	1613±247	1593±367	1317±418	296±114	N.D	392±78	163±78	172±47	172±47	382±156	397±162	218±49	218±49	382±156	397±162	218±49	218±49
4	2063±214	1901±461	1729±328	243±108	N.D	461±193	149±61	162±53	162±53	496±218	523±297	293±116	293±116	496±218	523±297	293±116	293±116
8	2416±608	2314±561	1923±216	212±93	93±12	482±213	109±45	116±35	116±35	559±296	591±213	217±63	217±63	559±296	591±213	217±63	217±63
24	2018±538	2102±391	1971±273	172±81	216±72	135±126	87±28	N.D	N.D	362±129	263±116	81±45	81±45	362±129	263±116	81±45	81±45
48	1046±291	967±218	1091±239	83±29	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
72	358±216	193±91	416±138	N.D	N.D	N.D	N.D	N>D	N>D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D

^a Results are expressed as ng/g fresh tissue or ml plasma (mean± SD) of 10 samples from five animals (two samples/animal).

^b DEET and permethrin were dissolved in ethanol.

Table.5 Tissue concentration^a of permethrin^b and metabolites following concurrent application of a single dermal dose of 1.3 mg/kg of permethrin and 400 mg/kg of DEET in Sprague-Dawley rats.

Permethrin							<i>m</i> -Phenoxybenzyl alcohol				<i>m</i> -Phenoxybenzoic acid			
Time (h)	Plasma	Liver	Kidney	Brain	Testes		Plasma	Liver	Kidney		Plasma	Liver	Kidney	
0.5	48±14	N.D	N.D	N.D	N.D		N.D	N.D	N.D		N.D	N.D	N.D	
1	87±29	N.D	N.D	N.D	N.D		N.D	N.D	N.D		N.D	N.D	N.D	
2	103±47	73±68	92±53	N.D	N.D		72±39	N.D	64±18		113±26	92±36	N.D	
4	123±62	102±37	136±29	57±34	N.D		101±23	78±61	83±22		108±47	106±24	N.D	
8	142±76	113±42	172±32	68±42	N.D		69±41	83±42	108±29		147±39	90±27	126±49	
24	187±56	78±29	111±28	59±31	N.D		52±13	71±26	127±53		97±19	83±42	107±23	
48	97±13	N.D	68±19	53±27	N.D		N.D	63±19	98±60		73±21	N.D	101±57	
72	N.D	N.D	N.D	N.D	N.D		N.D	N.D	N.D		N.D	N.D	N.D	

^a Results are expressed as ng/g fresh tissue of ml plasma (mean± SD) of 10 samples from five animals (two samples/animal).
^b Permethrin and DEET were applied in ethanol.

Table.6 Urinary excretion of metabolites^a of DEET^b following application of a single dermal dose of 400 mg/kg alone and in combination with 1.3 mg/kg of permethrin in Sprague-Dawley rats.

DEET alone						DEET in the presence of permethrin				
Time (h)	DEET	m-Toluamide	m-Toluic acid	Glucuro nide conjuga tes	Sulfate conjuga tes	DEET	m-Toluamide	m-Toluic acid	Glucuro nide conjuga tes	Sulfate conjug ates
0.5	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
1	53±8	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
2	98±13	N.D	67±9	N.D	65±13	62±15	N.D	N.D	N.D	N.D
4	128±21	68±12	96±13	134±20	112±18	91±15	N.D	65±112	73±9	101±20
8	189±23	79±11	119±20	197±17	139±26	137±20	68±14	83±17	89±11	123±21
24	227±46	103±17	168±14	287±24	268±32	194±35	94±18	118±21	135±22	190±15
48	232±41	108±21	174±23	349±27	397±43	204±17	101±26	151±16	178±30	218±23
72	234±45	112±23	178±21	352±38	412±30	205±12	106±18	154±18	186±21	237±28

Results are expressed as $\mu\text{g}/\text{mL}$

^a Results are expressed as µg/ml urine (mean±SD) of 10 samples from five animals (two samples/animal).
^b DEET and permethrin were applied in ethanol.

Table.7 Urinary excretion of metabolites^a of permethrin^b following application of a single dermal dose of 1.3 mg/kg alone and in combination with 400 mg/kg of DEET in Sprague-Dawley rats.

Permethrin alone						Permethrin in the presence of DEET					
Time (h)	permethrin	m-Phenoxybenzoic acid	m-Phenoxybenzoic alcohol	Glucuronide conjugates	Sulfate conjugates	permethrin	m-Phenoxybenzoic acid	m-Phenoxybenzoic alcohol	Glucuronide conjugates	Sulfate conjugates	
0.5	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
1	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
2	N.D	136±14	118±12	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
4	N.D	149±21	129±16	97±23	77±14	N.D	78±17	64±16	N.D	61±15	
8	N.D	173±22	153±22	158±16	153±29	N.D	97±13	95±17	169±30	159±14	
24	N.D	199±16	184±19	286±32	256±29	N.D	138±24	124±25	189±26	246±21	
48	N.D	201±19	191±18	309±23	295±36	N.D	169±25	178±23	196±17	299±22	
72	N.D	201±19	192±12	312±37	332±27	N.D	190±16	196±20	196±12	312±22	

Results are expressed as ng/ml urine (mean±SD) of 10

^a Results are expressed as ng/ml urine (mean±SD) of 10 samples from five animals (two samples/animal).
^b DEET and permethrin were applied in ethanol.

Table.8 Plasma concentration^a of permethrin^b and metabolites following application of intravenous, dose of 1.3 mg/kg, alone and in combination with 40 mg/kg of DEET in Sprague-Dawley rats.

<i>m</i> -Phenoxybenzyl alcohol				Permethrin in the presence of DEET		
Time (h)	Permethrin	<i>m</i> -Phenoxybenzyl alcohol	<i>m</i> -Phenoxybenzoic acid	Permethrin	<i>m</i> -Phenoxybenzyl alcohol	<i>m</i> -Phenoxybenzoic acid
0.5	1336±124	N.D	N.D	1763±109	N.D	N.D
1	1201±114	373±24	387±24	1638±92	251±22	226±23
2	1042±127	331±22	301±16	1401±81	219±14	208±12
4	932±51	308±19	251±16	1338±61	209±21	173±11
8	713±67	261±17	171±14	1123±41	158±16	132±12
24	629±49	223±13	129±15	927±32	130±14	108±14
48	363±23	53±9	63±15	503±42	61±10	78±9
72	191±12	N.D	N.D	292±19	N.D	N.D

^a Results are expressed as ng/g fresh tissue of ml plasma (mean± SD) of 10 samples from five animals (two samples/animal).
^b Permethrin was applied in ethanol while PB was applied in water.